The role of condensin in chromosome resolution

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Felix qui potuit rerum cognoscere causas

Happy is he who gets to know the reasons for things

Virgil, Georgics (c. 37BC)

Publications arising from this thesis

Condensin aids sister chromatid resolution by promoting their decatenation by topoisomerase II

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Facile synthesis of budding yeast a-factor and its use to synchronise cells of α mating type

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Declaration

I Adrian Charbin confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The condensin complex is a key determinant of mitotic chromosome architecture. In addition to its role in mitotic chromosome compaction, condensin is required for resolution of sister chromatid linkages during chromosome segregation in anaphase. How condensin resolves sister chromatids, and the nature of the chromosome bridges that are characteristic of cells harboring defective condensin, have remained topics of debate. Inactivation of topoisomerase II, the main enzyme that removes topological interlinks that persist between DNA replication products after their synthesis, leads to similar chromosome bridges. Here, we follow the catenation status of circular minichromosomes of three sizes during the *S. cerevisiae* cell cycle. Catenanes are produced in S-phase and, in part, they are readily resolved, aided by physical separation of sister chromatids during mitosis. Complete resolution, however, requires the condensin complex, a dependency that becomes more pronounced with increasing chromosome size. Condensin and topoisomerase II directly interact and, using purified proteins, we show that condensin stimulates DNA decatenation by topoisomerase II *in vitro*. Therefore, in parallel to promoting chromosome condensation, condensin facilitates topological resolution of sister chromatids to secure their successful segregation to daughter cells during cell division.

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Abbreviations

ARS	autonomous replicating origin
ATP	adenosine triphosphate
APC	anaphase promoting complex
BSA	bovine serum albumin
bp	base pairs
Cdk	Cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CPC	Chromosome Passenger Complex
Da	Dalton (kDa, kilodalton)
DAPI	4'-6'-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EM	electron microscopy
EDTA	ethylenediamine tetra-acetic acid
FACS	fluorescence activated cell sorting
FRAP	fluorescence recovery after photobleaching
FEAR	Cdc14 (Fourteen) Early Anaphase Release
FRET	fluorescence resonance energy transfer
G1	growth phase 1
GAL1	galactose inducible promoter 1
GFP	green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
Ig	immunoglobin
M phase	metaphase
MAT	mating type
MEN	Mitotic Exit Network
min	minute
noc	nocodazole

OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PEG	polyethylene glycol
PFGE	pulse field gel electrophoresis
PMSF	phenylmethane sulfonyl fluoride
rap	rapamycin
rDNA	ribosomal deoxyribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
S phase	synthesis phase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide electrophoresis
SMC	structural maintenance of chromosomes
TCA	trichloroacetic acid
topo I	topoisomerase I
topo II	topoisomerase II
ts	temperature sensitive
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
WT	wild type

Chapter 1. Introduction

1.1 Historical Perspective

The cell is the fundamental unit of all life we know, from single-celled bacteria through to the billions that you consist of, reading this thesis. Yet to describe cells as mere biological building blocks would be to overlook their immense sophistication. If there were any word in the English language whose simplicity most belies the complexity it represents, cell would be it. Since it was first discovered 350 years ago the cell has remained an object of great intrigue and even today, thousands of researchers internationally continue to make new discoveries about how cells work. One of the cell's most important, beautiful and poorly understood roles is that of cell division and it is this topic that this thesis addresses.

In 1665, the English philosopher and polymath Robert Hooke first discovered cells when examining thin slices of cork under a coarse, compound microscope. Seeing a multitude of tiny pores, he noted that they looked like the walled compartments a monk would inhabit in a monastery and so called these pores 'cells'. While Robert Hooke's simple microscope was able to see little more than cell walls, the compound microscope would be continuously improved upon during the 17th century. During this time, the accumulating observations of cells from naturalists, philosophers and the curious would eventually lead, in 1824, to Henri Dutrochet formulating one of the fundamental tenets of modern cell theory, by declaring that "the cell is the fundamental element of organization" (Nezelof, 2003). In the almost two hundred years that have passed since then, cell theory has become one of the foundations of biology. The theory states that all living things are made of cells, that cells are the basic building units of life and that old cells dividing into two creates new cells. While this latter point has been studied in great detail over the years, the mechanisms underlying cell division remain incompletely understood.

The first recorded observation of the internal organisation of cells during division came from the second half of the 19th century, when the German biologist Walther Flemming was using aniline dyes from a nearby chemical factory to stain

cells before examining them under the microscope. He observed a substance that strongly absorbed basophilic dyes, which formed apart of threadlike structures inside the cell nucleus; he would names these chromatin and chromosomes respectively. Through studying cell division, Flemming observed and carefully recorded (Figure 1.1) the distribution of chromosomes into the daughter cells, a process he called mitosis after the Greek word for thread. Following this, Flemming concluded for the first time that all cell nuclei came from another predecessor nucleus (Paweletz, 2001). Meanwhile, at roughly the same time, the Augustinian friar and scientist Gregor Mendel was studying heredity in pea plants, observing how his plants inherited certain traits with particular patterns. Unbeknownst to him, his observations would one day gain him fame as the founder of genetics. However, Flemming was unaware of Mendel's work, and the significance of Flemming's microscopy observations and their ability to describe a mechanism for inheritance would remain unrealised until the rediscovery of Mendel's studies almost two decades later in the early 1900's.

In the 20th century, it was shown that Flemming's chromatin was comprised of DNA and associated proteins, and that the DNA itself was the hereditary vehicle behind the process of inheritance Mendel had observed (Morgan, 1915). In turn, chromosomes would be fully described as organised chromatin structures, with each chromosome containing a single piece of DNA. However, it was quickly noticed that the presence of chromosomes was not a permanent feature in the cell. Indeed, during interphase when the cell is not undergoing cell division, individual chromosomes are not visible. Upon entry into mitosis, the distinct chromosome 'threads' could be seen forming through 'chromosome condensation' (Figure 1.2) a process driving the cytological manifestation of the chromosomes themselves. Thus, it became apparent that the appearance of distinct chromosomes was dependent on internal factors that were only active during specific times. This was supported by demonstrations where interphase cells were merged with mitotic cells causing condensation of the interphase DNA (Rao and Johnson, 1970).

Figure 1.1 Walther Flemming's observations of chromosomes during mitosis

Hand drawn illustrations of mitotic chromosomes, from Flemming's 1879 paper *"Beitrage zur Kenntniss der Zelle und Ihrer Lebenserscheinungen"*.

Figure 1.2 Mitotic cycle of rye (*Secale cereale*) chromosomes

Scanning electron microscopy (SEM) images of chromosomes undergoing condensation. In interphase (A), DNA is in an uncondensed state, such that individual chromosomes are not identifiable. When condensation begins (B), then individual chromosomes become visible. Condensation is greatest in late metaphase (E), when separation of chromatids becomes visible. After successful segregation of the sister chromatids (F), the chromosomes begin to de-condense. Images from (Zoller et al., 2004).

The compaction of chromatin that occurs when cells enter mitosis is probably the most iconic process of dividing cells and despite its seemingly superficial nature (Figure 1.2), chromosomes condensation is a ubiquitous process in eukaryotic cells. Such is the widespread prominence of condensation in the cell cycle that observers began to suspect that process goes beyond the task of just making chromosomes smaller. Indeed, it is currently hypothesised that chromosome condensation is required to resolve several important structural problems associated with proper DNA segregation in mitosis (Baxter and Aragon, 2012). Firstly and perhaps most obviously, chromosomes are longer than the length of the cell in which they reside. A typical human cell has a diameter of 10 micrometres, while the approximate length of DNA in its largest chromosome is 2 centimetres. Indeed the total length of cellular DNA is up to a hundred thousand times the length of the cell itself. Therefore if chromosomes are not properly condensed, they are unlikely to segregate properly, potentially becoming entrapped during cytokinesis (Koshland and Strunnikov, 1996a, Hirano, 2000). Secondly, interphase chromosomes mix, particularly in transcriptionally active areas and this needs to be separated during mitosis. Finally, after DNA replication is complete the two newly synthesised DNA strands, or sister chromatids, are extensively linked by both DNA intertwines, or catenanes (Sundin and Varshavsky, 1980), as well as proteinaceous links (Michaelis et al., 1997). These topological and proteinaceous links must be removed if the sister chromatids are to be successfully pulled apart to opposite poles of the cell by the mitotic spindle. The removal of all topological and proteinaceous links between sister chromatids is called chromosome resolution, a process describing the spatial individualization of the chromatids from each other. It has been proposed that chromosome condensation, an ordered process structuring DNA into individual rod-shaped chromosomes during mitosis, could drive the resolution of any catenation between chromatids as well as shortening their length, thus allowing their proper segregation during anaphase (Hirano, 1999).

Given its importance in chromatid resolution and segregation during mitosis, chromosome condensation can be unequivocally classified as an essential housekeeping function, indispensible for cell proliferation. However, the driver of condensation had not been discovered until recently. About 15 years ago parallel studies in several laboratories found that a protein complex called condensin was emerging as the primary, molecular driver of mitotic chromosome condensation

(Kimura and Hirano, 1997a, Sutani et al., 1999, Strunnikov et al., 1995a). It is condensin, with particular regard to its role in chromosome resolution, which will be the focus of this thesis.

1.2 The Structural Maintenance of Chromosome Protein Family

Condensin is a member of a family of proteins called the structural maintenance of chromosome (SMC) proteins. SMC proteins have come to be recognised as one of the most fundamental classes of proteins that regulate the structural and functional organisation of chromosomes from bacteria to humans (Ivanov and Nasmyth, 2005, Hirano, 2006). Given that SMC proteins arguably pre-date histones, it is not surprising that SMC complexes like condensin have adopted numerous roles throughout the course of evolution and have increasingly been viewed as global organisers of the genome (Hirano, 2006).

The best known of the SMC proteins is cohesin, a protein complex containing SMC1 and SMC3, which binds together sister chromatids from when they are created in S-phase, until they are destroyed at the onset of anaphase through cleavage by the protease seperase (Uhlmann, 2003). Sister chromatid cohesion forms the basis for the pairwise alignment of chromosomes upon the mitotic spindle, making possible the bi-orientated segregation of chromatids at anaphase (Tanaka et al., 2000, Toyoda et al., 2002). In addition to cohesin and condensin, a third SMC complex has been identified consisting of a SMC5/SMC6 heterodimer. This complex is least well characterised of the three, but it has been implicated in having a DNA damage prevention role as well as aiding DNA repair (Roy and D'Amours, 2011) In addition, it appears to be required for proper chromosome organisation during meiosis (Farmer et al., 2011).

Overall, the SMC family has been so interesting to chromosome biologists owing to several factors. First and foremost, the ubiquitous presence of these proteins, highly conserved across all kingdoms of life, suggest a pivotal and essential role for SMCs in the proliferation of cells. Genetic and cell-biology studies have shown that SMC proteins are involved in chromosome segregation, structure, regulation and recombinational repair, during both mitosis and meiosis. The next

intriguing feature of SMC proteins is their unique protein architecture. While originally supposed to be some form of chromatin motor, many lines of evidence have come to show that SMC proteins represent a completely novel form of protein machine, with a distinct tripartite ring structure, that function as a dynamic linker of the genome (Hirano, 2006).

1.2.1 Condensin function

While condensin has not been studied in as much depth as cohesin, multiple studies have shown that the condensin complex plays a crucial role in the compaction of DNA to form structurally stable mitotic chromosomes and their segregation *in vivo* (Strunnikov et al., 1995b, Hagstrom et al., 2002, Saka et al., 1994a, Hirano, 2005b, Ono et al., 2003, Steffensen et al., 2001, Hudson et al., 2003). The condensin complex is highly conserved among eukaryotes, and prokaryotes have a condensin–like complex as well. Condensin mutants in all eukaryotic model organisms, such as *S. pombe*, *C. elegans* or *D. melanogaster*, all display similar phenotypes of uncondensed nuclei and the presence of anaphase bridges (Bhat et al., 1996, Hirota et al., 2004, Saka et al., 1994a, Strunnikov et al., 1995b). In addition, the inactivation of prokaryotic SMCs in *E. coli, B. subtilis* and *C. crescentus* all show temperature-sensitive colony formation and an increase in the number of anucleate cells at the permissive temperature, suggesting a deficiency in chromosome segregation (Niki et al., 1991, Weitao et al., 2000, Moriya et al., 1998, Jensen and Shapiro, 1999).

Condensin has also been reported to have a range of roles in cellular functions during interphase, such as in the control of gene expression and heterochromatin formation (Bhalla et al., 2002) or in *X. laevis* CENP-A assembly (Bernad et al., 2011). Condensin plays a role in DNA replication checkpoint signalling in fission yeast (Aono et al., 2002) and additionally condensin mutants have defects in DNA damage repair (Akai et al., 2011).

1.2.2 Eukaryotic condensin structure

Condensin is a 650 kilodalton pentameric complex consisting of a heterodimer of SMC proteins as well as three additional non-SMC subunits (Figure 1.3). The first of the condensin subunits to be identified were proteins of the SMC family (Strunnikov et al., 1995b), including Cut3 and Cut14 proteins in *S. pombe*, their orthologs in *X. laevis* XCAP-E and XCAP-C and the Smc2 protein in *S. cerevisiae* (Hirano and Mitchison, 1994, Saka et al., 1994a, Strunnikov et al., 1995b). The SMC subunits form the core of condensin's enzymatic abilities, being both the DNA-binding subunits (Sutani and Yanagida, 1997a, Cheeseman et al., 2002) and the DNA-dependent ATPases (Kimura and Hirano, 2000). They have a unique domain organisation with two canonical nucleotide-binding motifs, known as the Walker A and B motifs, which are respectively located at the N-terminal and C-terminal domains. Between the two motifs are two long coiled-coil motifs that are connected by a non-helical sequence. Biochemical and electron microscopy studies have shown that the SMC monomer folds back on itself through antiparallel coiled-coil interactions, thereby creating an ATP-binding 'head' domain at one end and a 'hinge' domain at the other. The two SMC monomers can then associate with each other at the hinge domain to form a V-shaped molecule (Melby et al., 1998, Haering et al., 2002, Hirano and Hirano, 2002). Additionally, entire condensin and cohesin complexes from both *H. sapiens* and *X. laevis* have been visualised using electron microscopy. While cohesin displays an open and distinct ring conformation, condensin's ring structure appears much more closed with the arms emanating from the hinge at a smaller angle (Anderson et al., 2002) It has been hypothesised that these differences in structure could contribute to the different roles of condensin and cohesin *in vivo* (Hirano, 2005a).

Meanwhile, the non-SMC subunits are thought to have a regulatory role on the function of the condensin complex. *In vitro* studies using *X. laevis* egg extracts have shown that the non-SMC subunits of condensin modulate its ATPase activity (Kimura and Hirano, 2000), though an understanding of how they alter condensin activity and binding to chromosomes remains to be resolved. Of these three auxiliary subunits, the CAP-D2 and CAP-G contain HEAT (Huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A, TOR lipid kinase) repeats (Neuwald and Hirano, 2000). A number of chromosomal proteins contain these

repeats, which are thought to facilitate protein-protein interactions. The two HEAT repeat containing subunits are recruited by the third subunit, CAP-H (Brn1 in *S. cerevisiae*, Barren in *D. melanogaster*), which belongs to the kleisin family of proteins (Schleiffer et al., 2003). Kleisins are believed to act as bridging protein between the two SMC heads as supported by studies where the kleisin subunit was found to directly bind the SMC head domains of recombinant human condensin (Onn et al., 2007). For a schematic representation of the known condensin complexes in different model organisms, please see Figure 1.4.

Given that cohesin exists only as one complex across all organisms it is found in, the same was assumed for condensin for many years. However, a genome database search using the human CAP-D2 sequence revealed a distantly related protein, CAP-D3 (Ono et al., 2003). Immunoprecipitation of CAP-D3 revealed that it also associated with the core condensin subunits (SMC2 and SMC4) along with two other subunits, CAP-G2 and CAP-H2, the former containing HEAT repeats and the latter being a kleisin subunit. This new condensin complex was termed condensin II (Ono et al., 2003, Yeong et al., 2003) and while only higher eukaryotes have both condensin I and II complexes, the ratio of the two can vary significantly between different species. Phylogenetic analysis has offered some insight into how the two complexes might contribute to chromosome organisation. The model organisms *S. pombe, S. cerevisiae, A. nidulans* and *N. crassa* possess only condensin I and mitotic chromosome condensation is less dramatic a process in these species when compared to metazoans. This has led to the speculation that condensin II may allow organisms with larger chromosomes an additional level of organization (Hirano, 2005b). It has recently been shown using specific conditional knockouts of the two condensins that depleting either condensin results in differing mutant phenotypes. Super-resolution microscopy reveals that condensin I-depleted mitotic chromosomes are wider and shorter, with a diffuse chromosome scaffold, while condensin II-depleted chromosomes retain a more defined scaffold, with chromosomes more stretched and seemingly lacking in axial rigidity (Green et al., 2012).

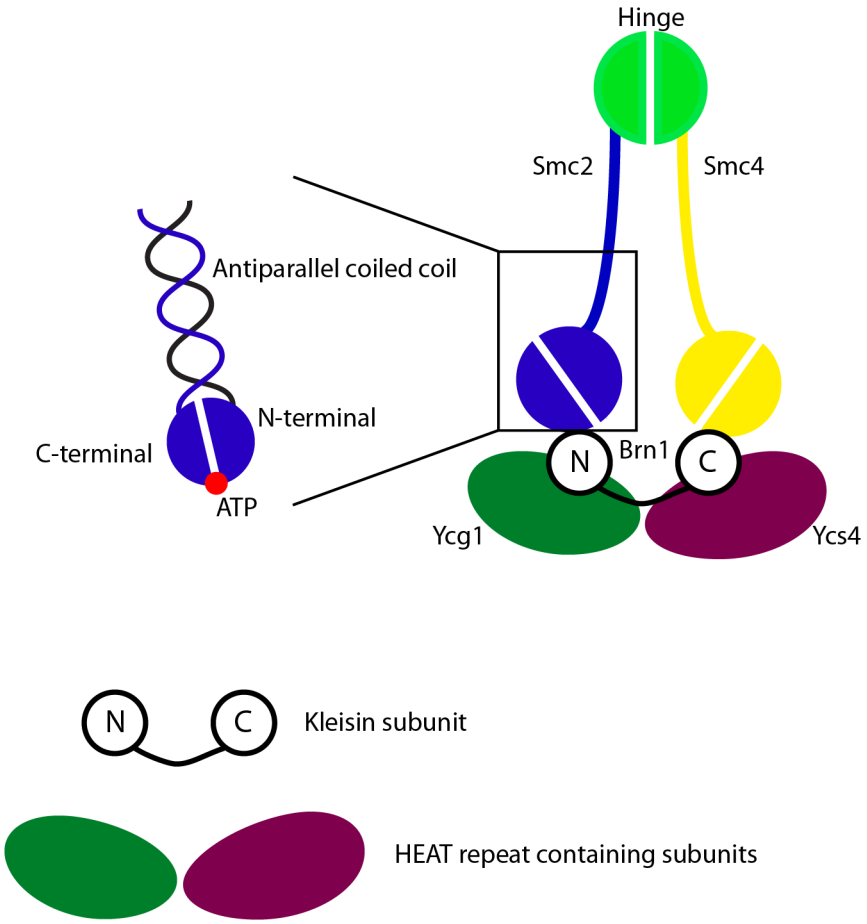

Figure 1.3 Schematic representation of the condensin complex

Condensin Subunits

	H. sapiens	*D. melanogaster*	*S. pombe*	*S. cerevisiae*
Core (I and II)				
SMC2	hCAP-E	DmSMC2	Cut14	Smc2
SMC2	hCAP-C	DmSMC4	Cut3	Smc4
I-specific				
HEAT	hCAP-D2	CG1911	Cnd1	Ycs4
HEAT	hCAP-G	CG17054	Cnd3	Ycg1
Kleisin	hCAP-H	Barren	Cnd2	Brn1
II-specific				
HEAT	hCAP-D3	CG31989	-	-
HEAT	hCAP-G2	?	-	-
Kleisin	hCAP-H2	CG14685	-	-

Figure 1.4 Condensin subunit comparisons among different organisms

1.2.3 Prokaryotic condensin structure

SMC proteins are widely conserved across the three domains of life. In prokaryotes, the best-studied homolog is the SMC-related protein MukB that functions as the core subunit of a bacterial condensin complex. It is found in a subclass of γ-proteobacteria that includes *E. coli*, and despite its limited sequence homology with other SMC proteins, MukB shares the five-domain structure common to all SMC family members consisting of a hinge domain, long coiled-coil arms, and head domains that form the ATP binding pockets (Melby et al., 1998). MukB forms the complete condensin complex along two other proteins, MukE, which binds the head domain of MukB via binding to MukF, the kleisin subunit that is remarkably conserved in bacteria (Yamazoe et al., 1999, Schleiffer et al., 2003). In *B. subtilis* the SMC protein also forms a condensin complex with two other subunits, ScpA and ScpB (Mascarenhas et al., 2002), and the SMC has an intrinsic DNA binding ability, distinguishable from cohesin by its ability to bind DNA in an ATP-independent manner (Hirano and Hirano, 1998a).

1.2.4 Condensin's *in vitro* biochemical activities

Condensin's ability to reshape chromosomes is both conspicuous and essential in the cell cycle. While there exist several hypotheses for the mechanistic basis of condensin's *in vivo* actions, which are discussed in greater length in chapter 1.3.1, several biochemical studies have revealed specific *in vitro* activities that presumably underpin condensin's *in vivo* functions.

Condensin is able to bind DNA independently of ATP hydrolysis (Kimura and Hirano, 1997b, Strick et al., 2004), which contrasts with cohesin where it is required for DNA binding (Onn et al., 2007). While purified condensin complexes only display low ATPase activity, this is stimulated by the addition of DNA, increasing ATP turnover rates by up to five-fold (Kimura and Hirano, 1997a, Kimura and Hirano, 2000, Yoshimura et al., 2002). Interestingly, this stimulation is dependent on the presence of the non-SMC subunits, both *in vitro* and *in vivo*, and is not seen in their absence (Kimura and Hirano, 2000, Stray and Lindsley, 2003). While all condensins possess ATPase activities, it remains unclear to what extent this activity underpins condensin's cellular functions.

In *X. laevis,* condensin isolated from mitotic cell extracts has the ability to promote the positive supercoiling of plasmid DNA in the presence of topoisomerase I (see chapter 1.5 for an introduction to topoisomerases) (Kimura and Hirano, 1997b). A similar activity was detected in a condensin fraction (specifically condensin II) purified from *C. elegans* embryos (Hagstrom et al., 2002). Visualisation by electron spectroscopic imaging suggests this supercoiling is driven by the wrapping of DNA around the condensin complex in two gyres (Bazett-Jones et al., 2002). Interestingly, condensin's supercoiling activity is not constant and phosphorylation appears to play a major role in its control. Several of condensin's non-SMC subunits are targets of mitotic kinases, notably Aurora B (Lavoie et al., 2004, Takemoto et al., 2006, Lipp et al., 2007) and cyclin-dependent kinase 1 (Cdk1) (Kimura et al., 2001, Kimura et al., 1998, Sutani et al., 1999). Additionally, a recent study in *S. cerevisiae* has shown that the Polo kinase Cdc5 directly phosphorylates all three regulatory subunits of the condensin complex *in vivo* and that consequently results in a hyperactivation of condensin's supercoiling activity (St-Pierre et al., 2009). Similarly, the MukB subunit of the *E. coli* SMC complex can support the formation of supercoils in circular plasmids in the presence of topoisomerase I. However, this reaction is ATP-independent and produces negative supercoiling, which is the opposite sign to that generated by eukaryotic condensin (Petrushenko et al., 2006).

A different change in DNA topology is observed when condensin holocomplexes immunopurified from *X. laevis* egg extracts or isolated *S. cerevisiae* Smc2/Smc4 dimers are incubated with nicked circular DNA in the presence of ATP and topoisomerase II. Here, the condensin complex converts the nicked circular DNA, via topoisomerase II-catalysed strand passage, into a positive three-noded knot, also known as a trefoil (Stray and Lindsley, 2003, Kimura et al., 1999). This indicates that condensin cannot only introduce positive supercoils into DNA, but has the ability to organise two or more supercoils into an ordered, solenoidal form. Intriguingly, this knotting activity may not require ATP hydrolysis, as evidenced by a mutant Smc2/Smc4 dimer, defective in ATP hydrolysis, which displays similar if not identical knotting activity (Stray and Lindsley, 2003). The *E. coli* MukB dimer, like its eukaryotic counterpart, can also promote the formation of right-handed DNA knots in the presence of topoisomerase II (Petrushenko et al., 2006).

An additional ATPase-independent activity is the promotion of single-stranded DNA annealing by the *S. pombe* Smc2/Smc4 heterodimer (Sakai et al., 2003, Sutani and Yanagida, 1997b). It has been speculated that this activity could be required to remove 'leftover' interphase products from mitotic chromosomes, such as RNA-DNA hybrids, in order to allow their correct segregation (Yanagida, 2000). The *B. subtilis* SMC dimer was shown to promote DNA reannealing in a similar but ATP-stimulated manner (Hirano and Hirano, 1998b).

1.3 Chromosome Condensation

Chromosomes are composed of roughly equal masses of DNA, histones and non-histone proteins. Depending on the cell cycle stage, chromosomes adopt different conformations with varying levels of compaction (see Figure 1.2). Mitotic chromosomes are the most distinct, reaching their most condensed forms just prior to anaphase onset. However, even in interphase when chromosomes are most 'uncondensed', their length is already 1,000 fold shorter than their linear counterparts would be. While the hierarchical packaging required to reach the level of compaction seen in mitotic chromosomes remains a hotly debated area in chromosome biology (Grigoryev and Woodcock, 2012), it is clear that condensation of interphase chromatin into mitotic chromosomes is tightly linked with cell cycle progression and requires condensin.

At the simplest level of packaging, DNA is organised by the histones. 147 base pairs of DNA wrap around each nucleosome, which are comprised of histones, and each nucleosome is linked to the next by approximately 60 base pairs of linker DNA (Laemmli et al., 1992). Unsurprisingly, histones were long-standing candidates for the molecular engine underlying chromosome condensation. However, when the histone fraction was extracted from mitotic chromosomes *in vitro*, the remaining insoluble non-histone fraction, the so-called 'chromosome scaffold proteins', appeared to maintain a structure similar to that of the intact mitotic chromosomes (Adolphs et al., 1977). While histones do contribute to condensation in protozoa and higher eukaryotes through phosphorylation of the core histone tail (Roth and Allis, 1992, Patterton et al., 1998), they are not responsible mechanically.

When high-salt extractions were performed on DNA to reveal what proteins were tightly associated with it, two of the most abundant components of eukaryotic chromatin were identified, topoisomerase II and condensin (Lewis and Laemmli, 1982, Earnshaw et al., 1985, Gasser et al., 1986). Soon after, topoisomerase II dysfunction in *S. pombe* cells was shown to disrupt both chromosome condensation and progression through anaphase (Uemura et al., 1987b, Holm et al., 1985), implicating topoisomerase II as an engine of condensation. However, topoisomerase II was also known to participate in other functions in chromatin, such as replication and transcription, and topoisomerase II's role in chromosome condensation was shown not to be universal (Hirano and Mitchison, 1994, Lavoie et al., 2002). Attention then focused onto condensin, a complex that when inactivated or depleted from eukaryotic cells results in reduced chromosome compaction (Saka et al., 1994b, Hirano and Mitchison, 1994, Strunnikov et al., 1995b, Koshland and Strunnikov, 1996b, Hudson et al., 2003, Steffensen et al., 2001). An overview of the mechanisms by which condensin could drive condensation can be seen in Figure 1.5.

1.3.1 Condensin's biochemical activities drive condensation

In *S. cerevisiae,* condensin binding is consistent throughout the cell cycle, implying that binding itself does not drive condensation, yet its activity is somehow maximised upon entry into mitosis (Guacci et al., 1994). As discussed in chapter 1.2.4, condensin can drive the supercoiling and knotting of DNA and these activities are modulated by cell cycle kinases. Indeed, condensin mutants that have a reduced ability to be phosphorylated *in vivo* are defective in anaphase-specific chromosome condensation (St-Pierre et al., 2009). Therefore, it would be reasonable to propose that the reconfiguration of chromosome topology caused by condensin's biochemical activities underpins chromosome condensation. Recent genome-wide mapping in *S. cerevisiae* has shown that condensin's individual binding sites have an average spacing of 420 kilobases of DNA between them (D'Ambrosio et al., 2008b). Consequently, between the binding sites of each condensin complex could be hundreds of nucleosomes that would introduce large numbers of negative turns to the DNA (Lee et al., 2007). This suggests that taken

on its own, condensin's biochemical activities would not be sufficient to obtain the required level of chromosome compaction in mitosis. Further experiments are required to test whether the density of condensin binding is greater on vertebrate chromosomes, which could account for their stronger compaction during mitosis. However, while condensin's biochemical activities may provide an essential contribution, it is more likely that condensin accomplishes condensation through a catalytic, linkage or co-operative mechanism.

1.3.2 Condensin as a catalyst driving condensation?

If condensin's biochemical activities alone are not sufficient to explain condensation then perhaps their action acts as mid-point, catalysing further topological changes by other chromosomal proteins such as the topoisomerases (see chapter 1.5). A recent study reported an increase in positive supercoiling of circular yeast mini-chromosomes preceding their segregation, and this is dependent on the presence of both the mitotic spindle and condensin function (Baxter et al., 2011). Most interestingly, they found that positively supercoiled mini-chromosome dimers isolated from topoisomerase II-deficient cells arrested in mitosis are more efficiently decatenated *in vivo* by recombinant topoisomerase II than negatively supercoiled dimers. This therefore suggests that condensin-driven changes in topology can render that DNA into a more favourable substrate for topoisomerase II. However, it is not immediately clear how condensin's promotion of decatenation by topoisomerase II could drive chromosome condensation and so far, there are no further reports of catalytic behaviour in condensin. Nevertheless, this particular behaviour could have substantial importance during chromosome resolution, a topic discussed in chapter 1.4.

1.3.3 Condensin could behave as a DNA linker to drive condensation

Another alternative is that condensin could compact DNA by acting as a molecular linker that brings different segments from within a single DNA strand. This idea is supported by an interesting experiment where a single DNA strand tethered to a paramagnetic bead was nano-manipulated allowing measurement of

its end-to-end extension and hence level of compaction (Strick et al., 2004). It was seen that condensin could rapidly physically compact DNA in an ATP-hydrolysis dependent manner. The compaction reaction was highly dynamic and reversible, with a kinetics that did not correspond with condensin-driven positive supercoiling. Rather, the behaviour is better explained through the contraction of the linear DNA by bringing two segments physically together and looping out the intervening DNA. Of course the proposal that one condensin complex can link two non-adjacent segments of DNA leads to the obvious question, is there more than one DNA binding site on the condensin complex?

Studies of eukaryotic condensin have so far only found evidence for one binding site. Atomic force microscopy of *S. pombe* condensin appears to show that DNA associates with the complex at the hinge domain of the SMC proteins (Yoshimura et al., 2002). Furthermore, *In vitro* assays have shown that the presence of DNA blocks the proteolytic cleavage of the Smc2 hinge domain (Onn et al., 2007) and furthermore, isolated Smc2/Smc4 hinge domains cause a shift in DNA electrophoretic mobility suggesting a direct interaction (Griese et al., 2010). In prokaryotes on the other hand, examination of their SMC proteins identified a positively charged patch in the structure of the dimerized head domains and well as in the hinge domain that could interact with DNA (Shin et al., 2009). The introduction of mutations that negate these positive patches reduce the shift seen in the electrophoretic mobility of plasmid DNA suggesting that for prokaryotic SMCs, the possibility remains that they can bind two different segments of DNA at their head and hinge domains.

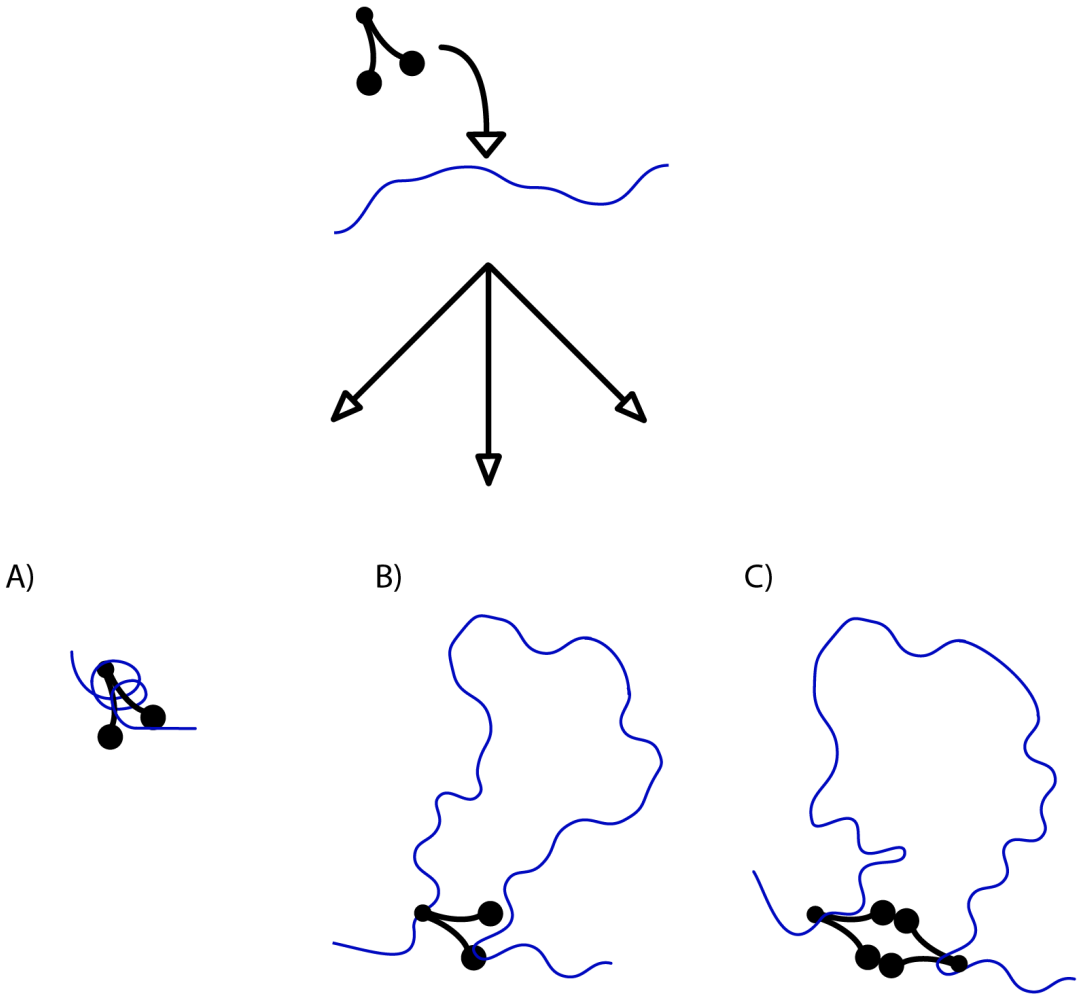

Figure 1.5 Possible mechanisms of condensation by condensin

A) Condensin's biochemical activity could drive condensation through changes in the topological conformation of the DNA it binds to
B) Condensin acting as a linker between distant segments of DNA
C) Condensin could interact with other condensin complexes to form multimers, thus bringing together different DNA segments

Is this head/hinge double binding of DNA by the condensin complex the only was it could link DNA? Despite condensin sharing a similar tripartite ring structure as its fellow SMC complex cohesin, it was assumed that it did not share cohesin's ability to capture DNA strands within its ring structure (Ivanov and Nasmyth, 2005, Anderson et al., 2002). However, using the same techniques used for cohesin, it has now been shown that condensin rings can also encircle chromosomal DNA (Cuylen et al., 2011). While cohesin rings may be able to freely slide along entrapped DNA, condensin binding has been mapped to distinct sites (D'Ambrosio et al., 2008b). If condensin entraps two DNA strands, this could be explained by the constant association of condensin with one chromosome segment, while local chromatin rearrangements are performed through the sliding of the entrapped second strand. Another possibility is that condensin complexes entrap one DNA strand, yet bring together separate DNA segments through association with other complexes.

1.3.4 Does condensin act through a co-operative mechanism?

Irrespective of how condensin complexes bind DNA, it has long been suspected that condensin function depends on interaction between the complexes. While it had been assumed that the engagement of SMC head domains always occurred in a heterotypic fashion (for example, Smc2-Smc4, but not Smc2-Smc2), the result that two Smc1 head domains had homodimerised in a protein crystal came as a surprise (Haering et al., 2004). While possibly an artefact of crystal preparation, it led credence to the idea that condensin complexes may function in a multimeric manner, with complexes associating to form co-operative condensation machinery. This proposal was further supported by single molecule studies where halving the protein concentration can completely eliminate condensation activity (Strick et al., 2004). Microscopy observations of condensin see its distribution along the inner axes of mitotic chromosomes, consistent with the notion that condensin forms part of a chromosome 'scaffold' (Maeshima and Laemmli, 2003, Cabello et al., 2001). However, how condensin complexes could multimerise remains unclear. Certainly if condensin 'scaffolds' were to form, they could not be static structures as shown by fluorescence recovery after photobleaching (FRAP) experiments, where

high turnover of condensin I was observed on mitotic chromosomes (Gerlich et al., 2006b, Oliveira et al., 2007). While evidence for multimerisation of eukaryotic condensins remains scarce, electron and atomic force microscopy has observed prokaryotic SMC complexes forming linear or rosette-like aggregates (Mascarenhas et al., 2005, Matoba et al., 2005).

In summary, of the possible mechanisms of condensin action discussed, it is most likely that reality combines aspects from all of them. Current research into condensin is revealing tantalisingly more about its mode of action, especially with regards to chromosome resolution. While playing a distinct role from condensation, it is becoming increasingly clear that condensin's contribution to both processes are inexorably linked.

1.4 Chromosome Resolution

Chromosome resolution is a critical step in mitosis if the sister chromatids are to be successfully segregated and thus correct chromosome segregation is a pre-requisite for preserving genome integrity. Cohesin and condensin are both required to ensure faithful chromosome segregation and dysfunction of either can result in mis-segregations and aneuploidies. While most aneuploidy human embryos are not viable, aneuploidies that occur later in human life are often associated with the development of malignant cancer. Chromosome instability, a term referring to a high rate of loss or gain of whole or parts of chromosomes, is a characteristic of most human cancers and is associated with their poor prognosis and drug resistance (McGranahan et al., 2012, Mannini et al., 2012). In summary, chromosome resolution describes a process of individualization, where all topological and proteinaceous links between the newly replicated sister chromatids are removed to produce distinct and spatially isolated entities.

1.4.1 Proteinaceous linkages

There are two types of link between the sister chromatids that need to be removed, the first being proteinaceous. These links are predominated by the SMC

complex cohesin, which establishes sister chromatid cohesin with a timing that is tightly coupled with DNA replication so as to not allow replication products to drift apart (Michaelis et al., 1997, Uhlmann and Nasmyth, 1998).

While cohesin binds to chromosomes before entry into S phase, it is only during DNA replication that cohesion is established. This is accomplished by a replication fork-associated acetyltransferase (Eco1 in *S. cerevisiae,* Eso1 in *S. pombe*) that acetylates the Smc3 subunit of cohesin. This protein is essential for the activation of cohesin and in cells lacking Eco1, cohesin bind the chromosomes, but the physical linkages between sister chromatids are never established (Ivanov et al., 2002). Cohesion establishment between sister chromatids is of utmost importance in mitosis, essential for the correct organisation and bi-orientation of the chromatids within the mitotic spindle in preparation for segregation during anaphase. Sister chromatid cohesion is lost upon anaphase onset through irreversible cleavage of the cohesin subunit Scc1 by the protease separase (Uhlmann et al., 1999). In vertebrates, the resolution of cohesion has an additional precursor step where a substantial portion of cohesin is removed from chromosomes as they condense in prophase in a step mediated by mitotic kinases (Losada et al., 2000, Losada et al., 2002, Sumara et al., 2000, Sumara et al., 2002). As soon as cohesin is dissociated from the chromosomes, the class I histone deacetylase Hos1 deacetylates the cohesin subunit Smc3 (Borges et al., 2010). As non-acetylated Smc3 is required as a substrate for cohesion establishment in the following G_1 phase, the cycle is completed and ready for the next round of DNA replication.

1.4.2 Topological linkages

Topological links describe the physical intertwining of two newly synthesised DNA strands produced during DNA replication. This occurs during DNA replication as a consequence of the convergence of replisomes. Initially, the topological challenge resulting from DNA replication was most obvious in prokaryotes, such *E. coli*, which have circular genomes. The unwinding of the parental DNA for replication in a closed, circular system would inevitably generate new strands of DNA that were linked, or catenated, together. To overcome this topological

constraint of DNA replication, it was first proposed in the 1960s that a nick in one of the parental DNA strands would eliminate any entanglements by allowing free rotation of the DNA along its axis (Cairns, 1963). While eukaryotic chromosomes are linear, it was found that these larger genomes behaved exactly like circular DNA owing to the existence of domains with fixed ends that prevent any free rotation of the DNA (Worcel and Burgi, 1972). From early on, predictions were made that in order for any DNA to be segregated post-replication, the DNA would need to be cut in several places (Tessman et al., 1957). These initial predictions were made with endonucleases in mind, but as it would turn out, endonucleases would not be responsible for these cleavages, but rather a new class of DNA enzymes called topoisomerases. Specifically, topoisomerase I would serve as a DNA swivel (Champoux and Dulbecco, 1972) while topoisomerase II would allow the physical passage of one DNA strand through another via a transient double-strand break (Brown et al., 1979, Gellert et al., 1976).

1.5 Topoisomerases

Topoisomerases are a very important class of cellular enzymes, required for the survival of all organisms through their ability to alter DNA topology by generating transient breaks in the double helix. There are two major classes of topoisomerases, type I and type II, which are distinguishable by the number of DNA strands that they cleave and the mechanism by which they alter the topological properties of the genetic material (Wang et al., 2002, Champoux, 2001, Deweese and Osheroff, 2009).

1.5.1 Types of topoisomerase

In eukaryotes, type I topoisomerases are monomeric enzymes that require no high-energy cofactors and can be organised into two subclasses: type IA and type IB. Type I topoisomerases alter topology by creating transient single-stranded breaks in the DNA, followed by passage of the opposite intact strand through the break (type IA) or by controlled rotation of the helix around the break (type IB). As a consequence of this process, type I topoisomerases can moderate DNA

supercoiling, but are not able to remove knots or tangles from duplex DNA (Leppard and Champoux, 2005). Eukaryotic type II topoisomerases function as homodimers and require divalent metal ions and ATP for complete catalytic activity. Topoisomerase II can interconvert different topological forms of DNA (also known as isoforms) via a 'double-stranded DNA passage reaction', which can be broken down into a series of distinct steps (Wang, 1998). Owing to this DNA passage mechanism type II topoisomerases cannot only alter DNA supercoiling like the type I enzymes, but also can remove DNA knots and catenation. It should be noted that after action upon by topoisomerase II, the chemical structure of the ligated DNA is identical to that of the original substrate. Hence, only the topological properties of the double helix are changed by the actions of this enzyme.

Lower eukaryotes and invertebrates, such as *S. cerevisiae,* encode only a single topoisomerase II (Goto and Wang, 1984), while vertebrate species encode two closely related isoforms of the enzyme, topoisomerase IIα and topoisomerase IIβ. While the isoforms are encoded by separate genes and differ in their protomer molecular masses (170 and 180 kDa, respectively), they share similar enzymological characteristics and high sequence homology. However, topoisomerase IIα and IIβ do have differing patterns of expression and their own distinct cellular roles. Type IIα is most similar to the type II topoisomerases see in lower eukaryotes and is essential for proliferating cells to survive, with protein levels increasing significantly during periods of cell growth. Furthermore, the enzyme is regulated through the cell cycle with peak concentrations in G2 and M phase (Heck and Earnshaw, 1986, Heck et al., 1988, Kimura et al., 1994). Topoisomerase IIα is associated with replication forks and remains tightly bound to chromosomes throughout mitosis. Thus, it is believed to be the isoform that functions in the cell's growth-related processes, such as DNA replication and chromosome segregation (Wang et al., 2002, Christensen et al., 2002). Topoisomerase IIβ on the other hand is dispensable on the cellular level and cannot compensate for the loss of topoisomerase IIα in mammalian cells, implying that these two isoforms do not play redundant roles in replicative processes (Sakaguchi and Kikuchi, 2004). Instead, topoisomerase IIβ appears to be required for proper neural development, most likely through involvement in the transcription of developmentally or hormonally regulated genes (Ju et al., 2006, Yang et al., 2000).

Prokaryotes encode two distinct type II topoisomerases, gyrase and topoisomerase IV. Gyrase is unique in that it is the only known topoisomerase that can actively negatively supercoil DNA and it plays an important role in regulating the superhelical density of bacterial DNA (Levine et al., 1998). Meanwhile, topoisomerase IV has a similar role to eukaryotic topoisomerase II, required for decatenation of sister chromatids after DNA replication and general removal of knots from the genome (Deweese and Osheroff, 2009).

1.5.2 Topoisomerase II mode of action

The ability of topoisomerase II to cleave and ligate DNA is central to all of its catalytic functions. To perform a strand passage reaction, topoisomerase II interacts with two DNA strands introducing a double strand break in one DNA strand, termed the gate or G segment, and then passing a second strand termed the T segment through the break (Figure 1.6). In the presence of Mg^{2+}, the enzyme can cleave the DNA by nucleophilic attack, forming a phosphotyrosine linkage between each single strand and a tyrosine in each subunit. The covalent enzyme-DNA linkage plays two critical roles in the topoisomerase II reaction mechanism. Firstly, it conserves the bond energy of the sugar-phosphate DNA backbone. Secondly, the covalent bond does not allow the cleaved DNA chain to dissociate from the enzyme, thus maintaining the integrity of the genetic material during the cleavage event (Wang, 1998). This is crucially important as double-stranded breaks in DNA can have mutagenic and even lethal consequences for cells.

The next step is ATP binding, which causes the enzyme to form a closed clamp. The closed clamp can also capture another strand (the T strand) that will then pass through the break made in the G strand. After passing through the break in the G strand, the T strand exits the enzyme through its carboxy terminus. ATP hydrolysis occurs at two steps in the reaction cycle, the first ATP hydrolysed likely assists in the passage of the T segment. The second hydrolysis step allows the clamp to reopen, releasing the G segment, although the enzyme may initiate another catalytic cycle without dissociating from the G strand (Harkins and Lindsley, 1998).

While it acts globally across all DNA, topoisomerase II appears to cleave at preferred sites. However, the consensus sequence for cleavage is weak, and many sites of action do not conform to it (Capranico and Binaschi, 1998). Currently, the mechanism by which topoisomerase II selects DNA sites to act upon is not apparent, and it is currently not possible to predict *de novo* whether a given DNA sequence will support scission (Deweese et al., 2008). Most likely, the specificity of topoisomerase II-mediated cleavage is determined by a combination of the local flexibility, structure or malleability of the DNA that accompanies the sequence, as opposed to the direct recognition of the bases that comprise that sequence (Velez-Cruz et al., 2005).

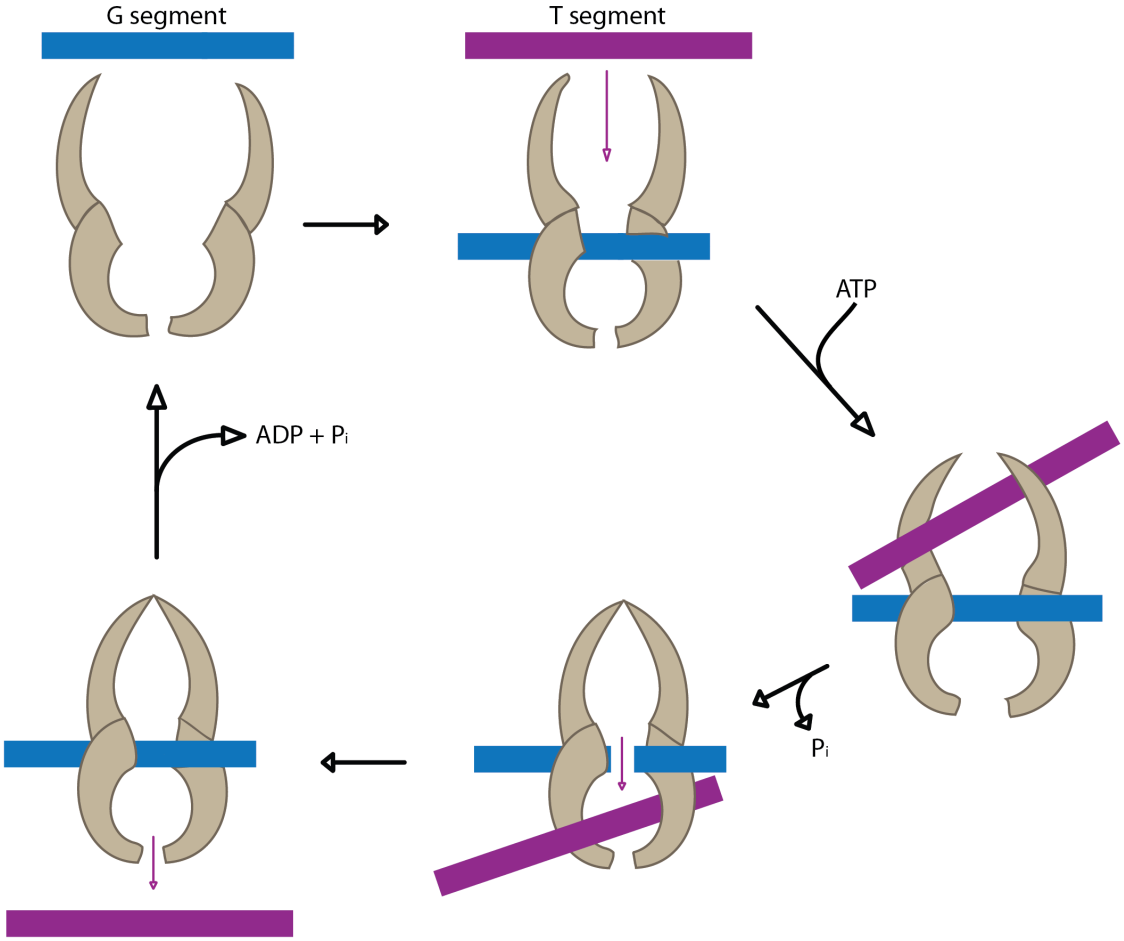

Figure 1.6 Topoisomerase II's mode of action

Type II topoisomerases bind two separate segments of DNA and then create a double-stranded break in one of the segments. After translocating the T segment through the cleaved nucleic acid 'gate' (G segment), the enzyme ligates the cleaved DNA, releases the translocated segment through a gate in the protein. Finally, the protein gate is closed and the enzyme regains the ability to start a new round of catalysis (adapted from Deweese et al., 2008).

1.5.3 The physiological importance of topoisomerase II

The correlation between faithful segregation of sister chromatids in mitosis and cancer cell development was touched upon earlier in this introduction. SMC proteins are required for proper chromosome segregation and their dysfunction can lead to the mis-segregations and aneuploidies associated with malignant tumours. However, the unique and critical role topoisomerase II has in managing DNA topology makes it perhaps the most important enzyme to understand with regards to chromosome segregation. Indeed, topoisomerase II has become a prime target for manipulation by anti-cancer drugs (Fortune and Osheroff, 2000, McClendon and Osheroff, 2007).

Unlike most other protein-targeted drugs, which kill cells by robbing them of an essential enzyme activity, topoisomerase-targeted drugs exploit the potentially lethal nature of topoisomerases. During the strand passage reaction, the topoisomerase II-DNA cleavage complexes are normally short-lived and readily reversible, with the DNA cleavage/ligation equilibrium of the enzyme greatly favouring ligation (Bender et al., 2008, Mueller-Planitz and Herschlag, 2008). Therefore, topoisomerase-targeted drugs 'poison' topoisomerase II by increasing the steady-state levels of the DNA cleavage complexes (Fortune and Osheroff, 2000, McClendon and Osheroff, 2007). This action converts topoisomerases into potent physiological toxins, resulting in the generation of DNA double strand breaks. Subsequently, the resultant mass of DNA damage can overwhelm the cell's repair pathways resulting in apoptosis (Roos and Kaina, 2012).

These drugs have proven to be powerful tools for oncologists, but growing evidence now suggests that topoisomerase II-mediated DNA cleavage can trigger chromosomal translocations that lead to the development of specific types of leukaemia. It has been shown that up to 3% of patients who take topoisomerase II-targeted drugs eventually develop acute myeloid leukaemia (Felix et al., 1995, Felix, 1998, Bender et al., 2008). Therefore, given topoisomerase II's role in both healthy cell division and the development of malignancies, gaining a better understanding of this enzyme's function in chromosome resolution and its relationship to other chromosomal proteins like condensin, is of great importance.

1.6 Chromosome resolution, topoisomerase II and condensin - an unresolved relationship

From bacteria to humans, DNA is globally underwound, or negatively supercoiled, by approximately 6% (Bauer et al., 1980). This state is important as duplex DNA is merely the storage form of the genetic information and in order to replicate or express this information, the two strands of DNA must be separated. As global negative supercoiling of the genome imparts increased single-stranded character to the double helix, it therefore also facilitates strand separation (Espeli and Marians, 2004). While negative supercoiling may promote many nucleic acid processes, DNA overwinding, or positive supercoiling, inhibits them. During replication the linear movement of DNA enzymes, such as the helicases and polymerases of the replisome, compresses the turns of the double helix into a shorter region ahead of the replication fork causing it to become increasingly overwound. The positive supercoiling that results makes it progressively more difficult to open the two strands of the double helix and eventually this can block essential nucleic acid processes (Travers and Muskhelishvili, 2007). Normally, type I topoisomerases are able to relieve this supercoiling but in the latter stages of replication when replisomes approach each other, the two replication forks impinge on each other and there is no longer room for a type I topoisomerase to relax the positive supercoiling (Murray and Szostak, 1985, Sundin and Varshavsky, 1980, Sundin and Varshavsky, 1981). By allowing the replisomes to rotate along the axis of the DNA strand, the torsion building up ahead of the fork is relieved. A consequence of this is that pre-catenated DNA molecules are created behind the fork, that upon replication completion, result in fully catenated DNA daughter molecules (Figure 1.7) (Zechiedrich and Cozzarelli, 1995, Lucas et al., 2001). These topological links between the sister chromatids must be removed should they be successfully segregated during anaphase and this is performed by topoisomerase II, the only enzyme capable of passing one DNA strand through another via a transient double strand break (Wang, 2002). Therefore, topoisomerase I and II mediated topological transitions at the replication forks ensure proper fork progression and stability and prevent activation of the DNA damage checkpoint (Bermejo et al., 2007). Topoisomerase II is therefore essential in mitosis and mutational analysis from various model organisms have shown it to

be the main decatenation driver, with mutants having distinct phenotype characteristics including poorly condensed chromosomes as well as non or mis-segregation of the sister chromatids in anaphase (Downes et al., 1991, Holm et al., 1985, Uemura et al., 1987a).

While the importance of topoisomerase II in decatenation was indisputable, several nagging issues persisted that questioned whether this enzyme could actually represent the whole story. The most pertinent question was how the cell could be sure its whole genome had been fully disentangled by anaphase onset? While topoisomerase II is an exceptional enzyme in its capabilities, its main shortcoming is that its actions are bi-directional. Not only can it remove topological linkages, but it can introduce them as well. In most circumstances, when two entangled DNA strands are decatenated by topoisomerase II, the liberated strands are then likely to move apart spatially. Not only are the two strands released from opposing sides of the topoisomerase enzyme, but their further separation is also energetically favourable through Brownian motion. Therefore, the likelihood that two strands come close enough together again to be recatenated is low. However, in a situation where the two strands are physically constrained, such as if a cohesin complex entraps both, then the probability of recatenation is greatly increased. Various arguments have been made to overcome this issue, the first being that since topoisomerases are ATPases, they are capable of catalysing an end point that is beyond a thermodynamically stable equilibrium (Vologodskii et al., 2001). In addition, it has been shown that topoisomerase II can detect specific DNA topology as suggested by their ability to interact with DNA crossovers (Dong and Berger, 2007, Zechiedrich and Cozzarelli, 1995). However, these arguments are not fully satisfactory in explaining how the complete decatenation of the sister chromatids is ensured.

The idea that condensin may contribute to resolution of sister chromatids in addition to its role in chromosome condensation was reinforced with the characterization of the complex in *S. cerevisiae* (Freeman et al., 2000). When compared to vertebrates, the chromosomes of budding yeast do not attain the same high levels of condensation in mitosis. In fact in *S. cerevisiae*, condensin-driven condensation causes only a 1.5-fold compaction of the chromosome arms and for successful chromosome segregation, only the longest chromosomes require this shortening (Guacci et al., 1994). Yet in the absence of condensin, all

the chromosomes fail to segregate (Bhat et al., 1996). This situation makes the sister chromatid sorting function of condensin even more apparent. Recently, studies of *S. cerevisiae* rDNA have been key in developing our understanding of condensin's resolution function.

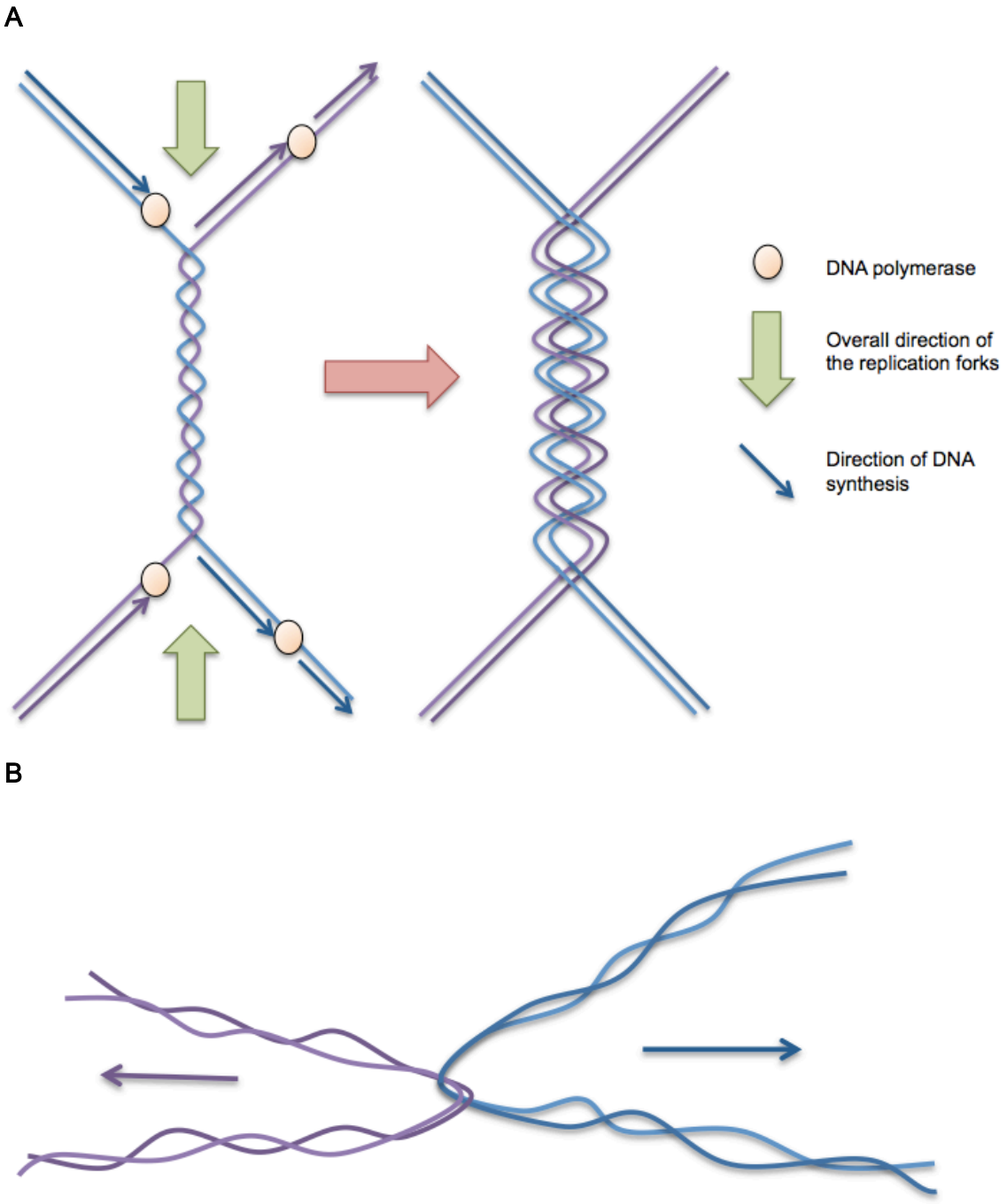

Figure 1.7 Schematic of colliding replication forks, and resultant catenation

A. As replication forks approach each other head on, helicases are no longer able to relieve the building superhelical tension in the DNA ahead of the fork. Through rotation of the replisomes this tension is relieved, but topological linkages, or catenation, is established between the sister chromatids.
B. A schematic of a topological link between DNA strands belonging to different sister chromatids. This link must be resolved to allow proper DNA segregation or else risk DNA damage.

1.6.1 Lessons from the rDNA locus

The budding yeast rDNA locus consists of between one and two hundred 9.1kb DNA repeats located on the longest arm of chromosome XII. At this site, ribosome biogenesis occurs as the nucleolus assembles on top of the locus. It has been previously noted that sister chromatids remain connected at the rDNA until mid-anaphase independently of the cohesin complex and that to remove this connection, both topoisomerase II and condensin activities are required (D'Amours et al., 2004, Sullivan et al., 2004). The phosphatase Cdc14, one of the most important down regulators of mitotic cyclins during mitotic exit in *S. cerevisiae*, appears to activate resolution of the rDNA locus, though a mechanistic explanation of this remains undetermined. The rDNA locus was interesting to study, as not only was it easy to visualise using immunofluorescence microscopy, but the condensin-mediated compaction of the locus could be functionally separated from condensin-mediated resolution. This is because compaction, but not resolution, depends on Aurora B kinase. Upon inactivation of the kinase, uncondensed rDNA sister chromatids succeeded in full rDNA disjunction (D'Amours et al., 2004, Sullivan et al., 2004). However, despite these uncondensed sister rDNA loci being fully resolved, they fail to be efficiently segregated in the dividing cell (Sullivan et al., 2004). Overall, this suggests that the most important function of condensin in rDNA resolution is not chromosome compaction, but resolution.

Examination of this situation is made even more interesting by considering the role rDNA plays within the cell. As the site of ribosome biogenesis, the rDNA locus is area of intense and continual transcription by RNA pol I and RNA pol III. It is well known that specific DNA structures are formed during the process of transcription and these are recognised and relaxed by topoisomerases (Mondal and Parvin, 2001, Osborne and Guarente, 1988). Besides this, in *S. cerevisiae* transcription continues non-stop through the cell cycle unlike in higher eukaryotes where it pauses during anaphase (Elliott and McLaughlin, 1979, Sullivan et al., 2004). Given that rDNA transcription generates a large number of DNA structural forms, it is conceivable that this locus would impede its own topological resolution without the concerted action of condensin and topoisomerase II. Correspondingly, it has been shown that growing *S. cerevisiae* cells in the presence of a transcription-inhibiting drug enhances rDNA segregation (Tomson et al., 2006). Given that the

requirement for condensin in rDNA partitioning and its massive enrichment at this loci (Freeman et al., 2000), it was therefore feasible that condensin's specific role was to aid resolution in topologically complex areas of high DNA transcription. However, a study then demonstrated that cell cycle down-regulation of rDNA transcription inversely correlated with condensin's binding efficiency to the rDNA repeats (Wang et al., 2006). Taken together, these studies painted a conflicting picture of what was happening. On the one hand, it seemed that high levels of transcription were conflicting with resolution, perhaps by preventing stable binding of condensin to the DNA. On the other, while transcription continues unabated at the rDNA loci, wild type cells can correctly segregate chromosomes during anaphase. This specific situation was finally clarified in a study where it was shown that the sister-rDNA segregation defect seen in condensin mutants could be overcome by ectopic expression of a foreign topoisomerase II (D'Ambrosio et al., 2008a). This result implied that it was indeed catenation preventing sister-rDNA segregation but that the endogenous *S. cerevisiae* topoisomerase II was ineffective in decatenating the locus without condensin.

Apart from *S. cerevisiae*, depletion of condensin in a range other cells resulted in phenotypes that closely resembled those seen in topoisomerase II inactivations (Strunnikov et al., 1995b, Bhat et al., 1996, Hagstrom et al., 2002, Hudson et al., 2003). These include poorly condensed and mis-segregated mitotic chromosomes, but the most striking feature are anaphase bridges (Figure 1.8). These result from the mitotic spindle pulling incompletely-resolved sister chromatids apart to opposing poles of the cell and the unresolved DNA is seen stretched as a bridge between the two spindle poles (Lavoie et al., 2002, Chan et al., 2007). The exact cause of these bridges has not been determined and their presence in condensin mutants has been attributed to many different causes, from the abnormal compaction of DNA in early mitosis (Hirano, 2005a) to the premature loss of compaction in early anaphase (Gerlich et al., 2006a, Vagnarelli et al., 2006). Conversely, the similarity between condensin and topoisomerase II inactivation phenotypes has led to the proposal that condensin may promote DNA decatenation through interaction with topoisomerase II (Bhat et al., 1996, Coelho et al., 2003, Sullivan et al., 2004). Despite the diverse range of proposals for condensin's role in chromosome segregation, the most likely mechanisms can be summarised in three different scenarios (Cuylen et al., 2011), discussed in the following three chapters.

Figure 1.8 Anaphase bridges in condensin mutants

Immunofluorescence microscopy images of anaphase bridges in DAPI-stained DNA presenting in (top) *S. cerevisiae* condensin mutants (Lavoie et al., 2002), and in (bottom) *H. sapiens* condensin mutants (Chan et al., 2007).

1.6.2 Condensin is required for complete removal of proteinaceous links

As introduced before, the main proteinaceous linker connecting the sister chromatids prior to anaphase is cohesin (see chapter 1.4.1). Therefore it is possible that depletion of condensin results in the incomplete removal of cohesin and this has been demonstrated in metazoan cells. In nocodazole-arrested HeLa cells depleted of condensin I (but not condensin II) still retain small amounts of cohesin on the chromosome arms (Hirota et al., 2004), impairing the resolution of chromosome arms normally observed under those arrest conditions, suggesting that condensin is required for complete removal. In *S. cerevisiae* condensin mutants, there is also an apparent failure to remove all cohesin from chromosome arms during mitosis (Renshaw et al., 2010) and even in meiosis, condensin mutants display telomeric segregation defects that can be reduced by over expression of seperase (Yu and Koshland, 2005). The kleisin subunit of cohesin is the cleavage target of separase and it has been shown that kleisin phosphorylation by PLK1 renders it a better substrate (Alexandru et al., 2001). Correspondingly, a reduction was observed in condensin mutants in the localization of PLK1 and the phosphorylation of cohesin's kleisin subunit during the first meiotic division (Yu and Koshland, 2005). However it is not clear if condensin could recruit PLK directly to cohesin, as while both SMC complexes are loaded onto the DNA by a shared loader complex (Scc2/Scc4) (Ocampo-Hafalla and Uhlmann, 2011), their final localization on the chromosome arms appear separate (D'Ambrosio et al., 2008b). Instead of a direct role, condensin could destabilise cohesin binding through the manipulation of mitotic chromosome structure. In support of this, the anaphase movement dynamics of fluorescently labelled *S. cerevisiae* chromosomes was tracked. Upon anaphase onset, segregation of the sister chromatids begins at the centromeres before moving along the chromosome arms with delays that increase towards the telomere. By promoting additional degradation of the kleisin at anaphase onset, and thus increasing cohesin removal, removes the observed delays. This implies that an uncleaved population of cohesin underlies the sequential stretching of DNA along the chromosomes arm. When this residual cohesin is cleaved by seperase, the chromosome arms spring back. However, in condensin mutants this elastic action is not seen. This leads to the proposal, which

has been supported by mathematical modelling, that condensin-dependent manipulation of DNA topology promotes removal of these residual uncleaved cohesins (Renshaw et al., 2010). Countering this, are studies done in human cells where depletion of Wapl results in much higher levels of cohesin being bound to the chromosomes. Despite the much higher levels of cohesin present, no segregation problems are observed (Shintomi and Hirano, 2009). As an interesting aside, there have also been reports that topoisomerase II functions in the cohesin cycle, hinting at the possible of a broad relationship between the enzyme and the SMC family (Tapia-Alveal et al., 2010).

1.6.3 Condensin directly partners with topoisomerase II to promote decatenation

DNA replication results in the topological linkage of the sister chromatids and this catenation is removed by topoisomerase II prior to anaphase onset. However, topoisomerase II is a bi-directional enzyme and as such the probability of these enzymes disentangling entire chromosomes on their own is very unlikely. As such, condensin has been proposed to promote of complete decatenation via a direct interaction with topoisomerase II. In prokaryotes, this proposal has recently received significant credit on the back of two studies demonstrating that the *E. coli* SMC protein MukB directly binds to and stimulates the activity of topoisomerase IV (Li et al., 2010, Hayama and Marians, 2010). *In vitro* assays demonstrated that the incubation of DNA with MukB prior to the addition of topoisomerase IV promoted the relaxation, and to a lesser extent decatenation, abilities of the enzyme. The site of interaction between the proteins was mapped via mutations to the MukB hinge domain and the C-terminal domain of the topoisomerase IV ParC subunit. This stimulatory effect could be a consequence of MukB binding simply boosting enzyme activity. Alternatively, MukB could preferentially bind sites of DNA catenation and then recruit topoisomerase IV to them.

Given the interaction observed in prokaryotes, it would not be unreasonable to predict a similar relationship in eukaryotes. Unfortunately, establishing where condensin directly interacts with topoisomerase II has been difficult and produced conflicting reports. The idea was first floated by the findings that mutations in the *S. pombe* genes encoding the Smc4 subunit and topoisomerase II are synthetic lethal

(Saka et al., 1994b). This was then followed by reports that the *D. melanogaster* kleisin subunit *Barren* co-localised with topoisomerase II and activated the enzyme's activity in *in vitro* assays (Bhat et al., 1996). However, while later studies confirmed that *D. melanogaster* cell extracts depleted of Smc4 lose their decatenation abilities, no direct interaction between condensin and topoisomerase II was observed (Coelho et al., 2003) and no direct interactions were observed in *S. cerevisiae* either (Bhalla et al., 2002). To further muddy the waters, extracts from mitotic *X. laevis* cells depleted of condensin showed no reduction in decatenation ability (Cuvier and Hirano, 2003). As such, there remains no overall consensus in eukaryotes on whether condensin direct binds topoisomerase II or not, with variation observed both between and within model organisms.

However, condensin could still directly promote decatenation through the recruitment of topoisomerase II to catenation sites on sister chromatids. This idea could explain the reduction in topoisomerase II staining on mitotic chromosomes spreads seen in *S. cerevisiae* condensin mutants (Bhalla et al., 2002). *In vitro* experiments show that condensins can stimulate plasmid knotting and preferentially bind structured DNA substrates, which hint at a possible affinity for DNA crossover sites (Kimura and Hirano, 1997b, Sakai et al., 2003). The enrichment of condensin in the topologically complex DNA landscape of rDNA loci in *S. cerevisiae* also lends credence to this line of thought (Freeman et al., 2000, Wang et al., 2005, D'Ambrosio et al., 2008b). However, the prospect of condensin acting as a recruitment agent for topoisomerase II remains unconvincing. Firstly, under some conditions the requirement for topoisomerase II function is no longer needed during rDNA segregation in anaphase, while condensin remains essential (D'Amours et al., 2004). While CHIP on CHIP mapping reveal a loose co-localization along the chromosomes (D'Ambrosio et al., 2008b), this is conflicted by immunofluorescence studies of mitotic chromosomes (Maeshima and Laemmli, 2003). Additionally, overexpression of topo II rescues co-localization in condensin mutants, but not function (Bhalla et al., 2002). Finally, the depletion of condensin subunits in metazoan cells does not significantly affect chromosomal topoisomerase II levels (Hudson et al., 2003, Hirota et al., 2004).

1.6.4 Condensin-driven reconfiguration of mitotic chromosome topology promotes decatenation

If condensin does not directly interact with topoisomerase II, it could still direct its behaviour. While condensin does not possess topoisomerase's ability to cut and translocate DNA strands, it does have its own biochemical activities that alter DNA topology as introduced in chapter 1.3.1. Condensin's reconfiguration of chromosome topology during chromosome condensation could promote decatenation and proper resolution on both local and global levels.

On a local level, it has been shown that positively supercoiled mini-chromosome dimers are more efficiently decatenated by topoisomerase II than negatively supercoiled dimers (Baxter et al., 2011). Given that the positive supercoiling of these mini-chromosomes is dependent on the presence of condensin (as well as the mitotic spindle), it suggests that condensin's supercoiling activity could generate DNA substrates, which are more amenable to decatenation, thereby pushing topoisomerase II's reaction equilibrium towards complete resolution. On a global level, the compacting activity of condensin within (but not between) mitotic chromosomes means that catenated sister chromatid DNAs are being pulled away from each other in a tug-of-war manner. Thus, it is not only energetically favourable for topoisomerase II to separate the DNAs, but upon decatenation, the sister chromatid DNAs are immediately physically isolated making any recatenation impossible.

Of course, condensin-driven condensation could also promote the types of resolution already discussed. For example, the stiffening of the chromatid fibre caused chromosome condensation could allow the transmission of mechanical forces generated at the centromeres by the pulling of the spindle out to the chromosome arms, thereby tearing apart any residual cohesin linkages that had not been cleaved by separase (Renshaw et al., 2010).

Condensin is required in three major mitotic events: chromosome condensation, chromosome resolution of proteinaceous linkages and chromosome resolution of topological linkages. It has become increasingly apparent that all three processes are intimately linked, with condensin being the common denominator. However, the relative contribution of condensin, particularly in regards to

chromosome resolution, as well the mechanism of its action remain unclear. This thesis presents work that aims to address these shortcomings in our knowledge of condensin.

1.7 An introduction to protein inactivations in *S. cerevisiae*

In the lab, we possess a range of temperature sensitive (*ts*) mutants suitable for use in functional studies and to date, the majority of what is known about yeast condensin has been learnt from the use of these *ts* alleles. In a *ts* mutant, a specific protein is altered such that at the permissive temperature, usually 25°C, the protein behaves as if wild type. However, upon temperature shift to the non-permissive temperature, usually 37°C, then the mutant protein will cease to function and be inactivated. At the start of our project, we used *ts* mutants extensively to inactivate proteins of interest and examine the consequence on catenation. Given the sizable collection of *ts* mutants already in possession by the laboratory, we did not need to create any further *ts* mutants in order to examine all the proteins we were interested in.

However, the use of *ts* mutants does present some problems. Firstly, some *ts* mutants have a leaky response to temperature shift, resulting in only a partial inactivation of the target protein. Secondly, not all the *ts* mutants are fully characterised, such that while we know the protein is inactivated at the non-permissive temperature, the exact mechanism of its inactivation is unclear. For example, if we shift a condensin *ts* allele, such as *smc2-8,* to a non-permissive temperature we are unsure of the exact effect on the protein. Does the shift cause the whole condensin pentameric complex to disassemble or does the complex remain intact but its functional activities are stopped? Does the protein remain bound to DNA and if so, could it still have some persistent effect on DNA topology, perhaps through interaction with another chromosomal protein? This lack of knowledge on the exact nature of the inactivation means that when we started contemplating the mechanisms behind our results, we were unable to have much confidence in the models we proposed.

Separate to this uncertainty about the inactivation, the temperature shift required to inactivate the target protein, usually a change from 25°C to 37°C, will of

course itself have an effect on the cells (Morano et al., 2012). At the higher temperature, the cell's stress response will be activated and when considered in the light of our catenation assay, we needed to be sure that the sometimes-subtle changes observed in our data were a result of a specific target protein inactivation and not the cell's innate stress response. While we performed the necessary control experiments (see chapter 3.2.2 of the results) at the start of our project, these limitations were deemed acceptable because the only other alternative for inactivating target proteins was the Degron method (Dohmen et al., 1994), but this too uses a temperature shift to initiate degradation.

However, shortly after starting this PhD project, a paper was published detailing a novel technique for the specific depletion of a protein of interest from the nucleus of *S. cerevisiae* called anchor away (Haruki et al., 2008b). The concept was simple: to sequester, in a ligand-dependent manner, a gene product of interest from its functional compartment to a different one, where it is anchored to a suitable protein receptor (the anchor) and cannot exert its function. Thus, a target protein could be specifically depleted from the nucleus by being transported out of the nucleus and tethered to a receptor protein located in the cytoplasm. This was achieved through the use of the human 12kDa, FK506 binding protein (FKBP12) and the 11kDa, FKBP12-rapamycin-binding (FRB) domain of human mTOR, which were fused to the anchor and target proteins, respectively. Rapamycin binds to the FKBP12 domain where it forms an interaction surface for the FRB domain to establish a tight tertiary complex of nanomolar dissociation constant (Chen et al., 1995). By taking advantage of the massive flow of ribosomal proteins through the nucleus during maturation, when rapamycin is added and there is formation of a tertiary complex composed of the anchor, rapamycin, and the target, then this results in the rapid depletion of the target from the nucleus (Figure 1.9).

The new anchor away technique was exciting as it provided a new means by which to inactivate a particular protein, in this case through the specific depletion of the target protein from the nucleus. Given the problems arising from use of *ts* alleles, using anchor away offered many advantages, the most important being that any temperature shifts could be avoided and all experiments performed at 25°C. Regarding the efficiency of the depletion, by probing Western blot samples of chromatin pellets, we observed an approximately 80% depletion of the target protein thirty minutes after rapamycin addition.

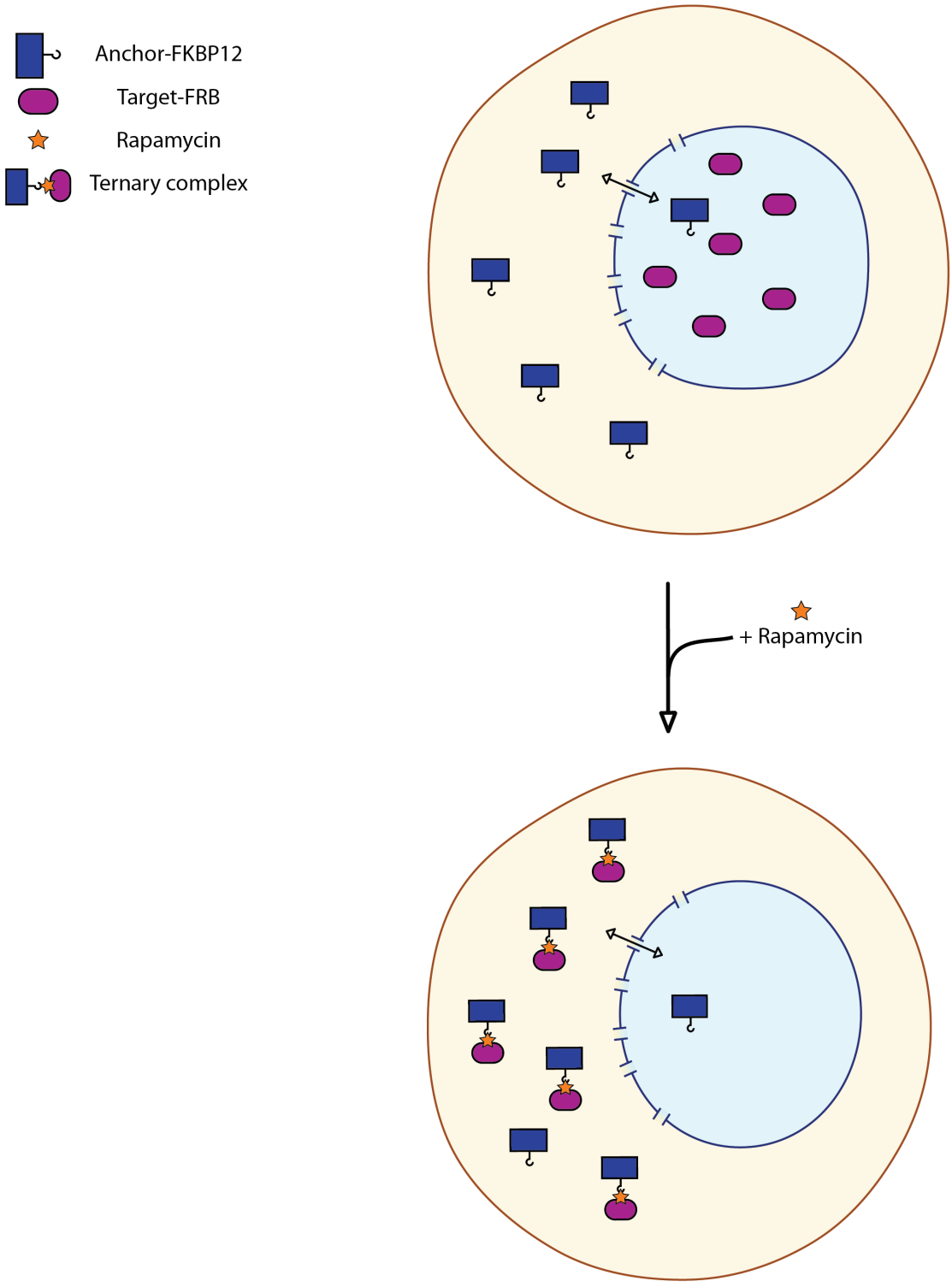

Figure 1.9 The anchor away technique of nuclear depletion

This simplified schematic shows how the addition of rapamycin results in the formation of a ternary complex including the target protein with the epitope tag FRB and the (non-nuclear) anchor-FKBP12. The end result is the depletion of the target protein from the cell's nucleus.

Therefore, when presenting our results we have often done the inactivation of the protein using both *ts* alleles and the anchor away technique and this is because of several reasons. Firstly, by the time we had obtained the anchor away strain in the lab we had already done many of experiments using the *ts* alleles. Later on in the PhD, it would often be faster to do experiments using the *ts* strains as it could take time to correctly tag or cross strains for use with anchor away. Furthermore, and perhaps most importantly, by repeating key experiments using both approaches we were able to really ensure that the resulting phenotypes observed were indeed a consequence of inactivating that specific protein and not a side effect of temperature, strain sickness or incomplete inactivation of the target protein. As such, it acted as a control and confirmation of our observations and thus both approaches were used throughout.

Use of the anchor away strain also resulted in a small side project. In research labs using *S. cerevisiae* as their model organism, the ability to synchronise cell populations using the mating pheromone **α**-factor has proven invaluable, especially for cell cycle studies. Given its common use, the **α**-factor response pathway has also become an important model to study the molecular mechanism of G-protein coupled receptor signalling (Vallier et al., 2002). However, only cells of the **a** mating type will respond to this pheromone. As it happened, the anchor away cell lines were of the **α** mating type, and as such do not respond to **α**-factor but rather to **a**-factor, a farnesylated and C-terminally methylated 12 amino acid peptide. Because of its more difficult chemical synthesis, **a**-factor is not readily available and consequently the **a**-factor response in *S. cerevisiae* is poorly characterised.

Given our extensive use of anchor away strains throughout this project, and resultant high consumption of **a**-factor, we collaborated with the peptide synthesis laboratory in our institute. While they developed a new and improved strategy for **a**-factor production based on solid-phase peptide synthesis, we characterised the successful use of the resultant **a**-factor in synchronization of cell populations (O'Reilly et al., 2012). Results from this characterization of **a**-factor and comparisons with **α**-factor can be seen in chapter 6 of the results.

Chapter 2. Materials & Methods

2.1 Yeast growth and manipulation

2.1.1 Yeast strains

The strains used in this study were of the W303 background, or were backcrossed against this background, with the exception of the *brn1-9* (Y3939) and *smc4-1* (Y3954) strains that were of S288c background. Strain details are listed below:

Strain Number	Genotype
K699	*MAT***a**, *ade2-1, trp1-1, can1-100, KAN, his3-11, ura3-52, GAL, psi+,* (w303 wild type)
Y216	*MAT***α**, *SCC1- HA_6::HIS3*
Y390	*MAT***a** *ATa, BRN1-HA_6::HIS3*
Y2665	*MAT***a**, w303 wild type containing *pS14-8(LEU2)*
Y3277	*MAT***a**, *TOP2-PK_3::LEU2*
Y3278	*MAT***a**, *BRN1-HA_6::HIS3, TOP2-PK_3::LEU2*
Y3939	*MAT***a**, *brn1-9, pS14-8(LEU2)* (S288c background)
Y3940	*MAT***a**, *NET1-GFP::TRP1, ycg1-10, pS14-8(LEU2)*
Y3954	*MAT***a**, *smc4-1, pS14-8(LEU2)* (S288c background)
Y3976	Topo II purification strain. See Worland & Wang 1989
Y3993	Condensin purification strain. See St-Pierre et al. 2009
Y4007	*MAT***a**, *top2-4, pS14-8(LEU2)*
Y4029	*MAT***a**, *scc1-73, pS14-8(LEU2)*
Y4031	*MAT***a**, *SCC1-HA_6::HIS3, TOP2-PK_3::LEU2*
Y4113	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, smc2-FRB::HIS3, MET3pr-HA-CDC20::URA3, pS14-8(LEU2)*
Y4129	*MAT***a**, *MET3pr-HA-CDC20::URA3, pS14-8(LEU2)*
Y4201	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, scc1-FRB::HIS3, pS14-8(LEU2)*
Y4059	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, brn1-*

	FRB::HIS3, pS14-8(LEU2)
Y4199	*MAT***a**, *pRS316(URA3),* (S288c background)
Y4235	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, scc1-FRB::HIS3, smc2-FRB::kan^R, pS14-8(LEU2)*
Y4236	*MAT***a**, *smc2-8, pRS316(URA3)*
Y4333	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, brn1-FRB::HIS3, pRS316(URA3)*
Y4259	*MAT***a**, *leu2Δ::kan^R, RCIII-SUP11-LEU2-3ARS*
Y4264	*MAT***a**, *leu2Δ::kan^R, brn1-9::TRP1, RCIII-SUP11-LEU2-3ARS*
Y4327	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, leu2Δ::kan^R, brn1-FRB::HIS3, RCIII-SUP11-LEU2-3ARS*
Y4332	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, pS14-8(LEU2)*
Y4333	*MAT***α**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, brn1-FRB::HIS3, pRS316(URA3)*
Y4334	*MAT***a**, *tor1-1, fpr1::NAT, RPL13A-2xFKB12::TRP1, top2-FRB::HIS3, pS14-8(LEU2)*

2.1.2 Yeast strain creation, mating and tetrad dissection

Strains were constructed by transformation with the appropriate DNA integration fragment designed for gene knockout or tagging. Affinity epitope tags were fused at the gene endogenous loci for Western blot detection, or a 3xGFP and mRFP cassette for detection by fluorescent microscopy, using polymerase chain reaction products (Bähler et al., 1998, Knop et al., 1999). In order to cross to strains, mating was induced through incubation of opposite mating type yeast strains on YPD plates at 25°C for at least 6 hours. Diploids were then selected by re-plating the crossed strains onto appropriate selective media and grown again on YPD overnight. Diploid cells were sporulated on sporulation media (100mM CH_3COONa, 20 nM NaCl, 25 mM KCl, 1.5 mM $MgSO_4$ and 1.5% w/v agar) until tetrads appear. To break their asci, spores were treated with Zymolase T-20 (MP Biomedicals) for 10 minutes at 30C. From each ascus, four spores are released which were then dissected using a Singer-MSM micromanipulator. Finally, spores

are incubated at 25°C until colonies formed and from these colonies can then be re-streaked onto the appropriate selective media to determine the genotypes of the progeny generated.

2.1.3 Yeast media, cultures and synchronizations

Cells were grown in YP supplemented with 2% w/v glucose (YPD) or 2% w/v raffinose/galactose (YP-Raff/Gal). Yeast cells of **a** mating type were arrested in G_1 with the mating pheromone **α**-factor. To arrest cells in G_1, early log phase cultures (OD600 = 0.1) was treated with 0.5 µg/ml **α**-factor (provided by peptide services, Cancer Research UK). Cells of **α** mating type were synchronised using 0.04 µg/ml **a**-factor (provided by peptide services, Cancer Research UK), as described (O'Reilly et al., 2012). One hour after the initial addition of **α**-factor or **a**-factor, the same amount is added to the culture. Within two hours complete synchronization of the culture in G_1 is achieved. Cell cycle arrest was determined both cytologically by the appearance of a characteristic 'shmoo' and by FACS analysis of DNA content. G_1 arrested cells were collected on a membrane filter (Schleicher & Schuell, ME28, 1.2mm) using a filtration apparatus (Millipore). Cells were washed with at least five times the initial culture volume of YPD before being release into fresh YPD media.

For arresting cells at metaphase, 5 µg/ml nocodazole was added to the culture from a 2 mg/ml stock solution in DMSO. To metaphase arrest strains using the repression of *MET*-Cdc20, cells were grown in YNB supplemented with 2% glucose. To induce arrest, 2 mM methionine was added to the culture.

2.1.4 Anchor away strains and nuclear depletion

Anchor-away strains were created by FRB-tagging of the target gene in the anchor away strain background as described (Haruki et al., 2008b). To deplete nuclei of cohesin or condensin, rapamycin was added to the respective anchor away strains at the time of their release from synchronization in G_1.

2.1.5 Yeast transformations

Transformation of cells with plasmids, minichromosomes or PCR products was performed using standard lithium acetate transformation. In brief, 50 ml of mid-log phase culture was pelleted at 3,000 rpm for 5 minutes. The cell pellet is washed with 1 ml distilled water, then washed with 1 ml 1X TEL (10 mM Tris/HC pH 7.5, 0.1 mM EDTA, 100mM Lithium Acetate) before being re-suspended in a final volume of 100 µl 1X TEL. 1 mg of either linearised DNA vector or PCR product was mixed with 2 ml of a 10 mg/ml single stranded carrier DNA from salmon sperm and 600 µl TELP (TEL plus 40% PEG 3350 or 4000). 50 µl of the cell suspension in TEL was then added to this mix and vortexed for 10 seconds. The mixture is incubated at 25°C for 4 hours and afterwards heat shocked at 42°C for 15 minutes. The cells are pelleted at 6,000 rpm for 2 minutes, washed with and re-suspended in 1M sorbitol before being plated onto YNB agar plates lacking the auxotrophic amino acid used for selection. Transformants were checked for correct integration via either western blot analysis, Southern blot analysis or in the case of anchor away strains, by death on rapamycin containing media.

2.2 General molecular biology techniques

All standard molecular biology techniques such as PCR, restriction enzyme endonuclease digestion or bacterial plasmid purification, were carried out as described in (Sambrook and Gething, 1989) or as per the supplied manufacturers protocol.

2.3 Protein analysis techniques

2.3.1 Protein extract preparation

Cell extracts were prepared using the TCA method. 5-10 ml of mid-log phase cell culture is collected and cells pelleted by centrifugation, 3,000 rpm, 5 minutes at 4°C. Cells are resuspended in 1 ml of 20% trichloroacetic acid (TCA) and kept on ice until the end of the time course. Cells are spun down for 1 minute at 13,000 rpm, then washed with 1 ml of 1M Tris-Base before being resuspended in

100 µl 2X SDS-PAGE loading buffer containing DTT. Samples are boiled for 2 minutes at 95°C, then 100 µl of 0.5mm glass beads (BioSpec Products, Inc) were added and the cells broken using a FastPrep FP120 cell breaker (Bio101). To separate the cell lysate from the glass beads, a few small holes are made in the bottom of the cell breaker tube using a 27G needle and the tubes are placed inside 15 ml Falcon tubes before being spun at 3,000 rpm, 5 minutes at 4°C. The collected lysate is then boiled at 95°C for 5 minutes and cleared by centrifugation at 13,000 rpm for 5 minutes before being loaded onto an acrylamide gel.

2.3.2 Chromatin pellets

Collect 50 ml of mid-log phase culture and centrifuge at 3,000 rpm for 5 minutes at 4°C to pellet cells. If performing timecourse, resuspend cells in 50 mM HEPES/KOH pH 7.5, 100 mM KCl, 1.5 mM $MgCl_2$, 1 M sorbitol and keep on ice. When time course is over and samples can be processed, resuspend cells in 3 ml of 100 mM PIPES/KOH pH9.4, 10 mM DTT, 0.1% Na-Azide) and incubate for 10 minutes at room temperature. Then spin the cells for 2 minutes at 2,000 rpm and aspirate supernatant. Cells are resuspended in 2 ml of 50 mM KPi pH 7.4, 0.6 M sorbitol, 10 mM DTT and to each sample 4 µl of 20 mg/ml Zymolyase 100T (MP Biomedicals) is added for spheroplasting of the cells. After spheroplasting, all work should be done at 4°C. Cells are spun down for a minute at 4,000 rpm and then washed with 1 ml of 50 mM HEPES/KOH pH 7.5, 100mM KCl, 2.5 mM $MgCl_2$, 0.4 M sorbitol and then spun again for a minute at 4,000 rpm. Cells are resuspended in an equal volume of buffer EB (50 mM HEPES/KOH pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 20 µg/ml leupeptin, 2 mM benzamidine, 2 µg/ml aprotinin, 0.2 mg/ml bacitracin, 2 µg/ml pepstatin A and 1 mM PMSF). To this 10% Triton X-100 is added to a final concentration of 0.25% and then incubated for 3 minutes on ice with occasional vortexing, which produces the whole cell extract. Prepare 100 µl EBX-S (EB + 0.25% Triton X-100 + 30% sucrose) in separate Eppendorf tubes and lay 100 µl of the whole cell extract onto the EBX-S. Spin for 10 minutes at 12,000 rpm, which will produce a white chromatin pellet, a clear sucrose layer and above this, a yellow supernatant fraction.

2.3.3 SDS-PAGE electrophoresis and western blotting

Protein samples were resolved on acrylamide/bis-acrylamide (37.5:5:1, amresco) 375 mM Tris-HCl pH 8.8 and 0.1% SDS. Small proteins of less than 30 kDa were typically resolved on 10-12% gels and larger proteins over 100 kDa on 8% gels. A stacking gel was used on top of the separating gel and consisted of 125 mM Tris-HCL pH 6.8, 5% acrylamide/bis-acrylamide and 0.1% SDS.

For electrophoresis a current of 50 mA was applied in an electrophoresis tank (CBS Scientific) using SDS-PAGE running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS). To follow electrophoresis progression, and to allow the later size comparisons of proteins on the gel, a broad range pre-stained protein marker (New England Biolabs) was used.

After completion of electrophoresis, proteins were transferred onto pre-equilibrated nitrocellulose membranes (GE Lifesciences) using a wet-transfer tank (Biorad) and transfer buffer (3.03 g/l Tris base, 14.1 g/l glycine, 0.05% SDS and 20% w/v methanol). To check that the transfer was effective, the membrane is stained with Ponceau S solution (Sigma). Then the membrane is blocked for an hour with a 5% milk solution (Marvel) in PBST (170 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.01% Tween 20) at room temperature. Following this, membranes were incubated with primary antibodies diluted in milk solution for one hour at room temperature, or overnight in the cold room. Primary antibodies used and their final concentrations were a-Pk clone SV5-Pk1 (1:5000, Serotec), a-HA clone 3F10 (1:5000, Roche), a-HA clone 16B12 (1:5000, Covance) and a-tubulin clone YOL1/34 (1:1000, Serotec). After primary antibody incubation, membranes were then washed in an excess of PBST for 30 minutes. Horseradish peroxidase (HRP) coupled secondary antibodies (anti-mouse or anti-rabbit, 1:5000, Amersham) were then incubated with the membrane in PBST containing 5% milk for a further hour. Again membranes were then washed three times with an excess of PBST for 30 minutes before developing with ECL (Amersham) according the manufacturer's instructions.

2.4 Mini-chromosome purification, electrophoresis and catenane quantification

1 gram of cells were collected at each time point, resuspended in ice cold 1 M sorbitol, 0.1 M EDTA pH 7.5, 0.02% sodium azide, and kept on ice until the end of the timecourse. Cell pellets were then resuspended in 0.5 ml of the same buffer, 20 µl of 2.5 mg/ml Zymolyase 100T (MP Biomedicals) was added and the suspension incubated for 1 hour at 37°C with mild agitation. Cells were collected by centrifugation for 10 minutes at 13,000 rpm, supernatants removed by aspiration and the pellets resuspended in 0.5 ml of 50 mM Tris/HCl pH 7.4, 20 mM EDTA. SDS was added to a final concentration of 1% and samples were incubated at 65°C for 15 minutes. Then, 0.2 ml of 5 M potassium acetate was added and the samples placed on ice for 1 hour. The precipitate was removed by centrifugation for 5 minutes at 14,800 rpm and the supernatants collected. The centrifugation step was repeated until the supernatant fractions were clear of any debris. 2 volumes of 100% ethanol were now added, and after 5 minutes the DNA was collected by centrifugation for 1 minute at 13,000 rpm. The supernatants were aspirated and the pellets allowed to air dry. Once dry, pellets were carefully resuspended in 0.3 ml of TE, pH 7.4 and 50 mg/ml RNase A added and incubated for 30 minutes at 37°C, before addition of 100 mg/ml proteinase K for further 30 minutes. Now, 12.6 µl of 5 M sodium chloride was added, and the DNA precipitated by addition of 0.63 ml 100% ethanol. Samples were again centrifuged, supernatants aspirated and the pellets air-dried. After resuspension in TE, the DNA concentration was measured (NanoDrop, Thermo Scientific) and adjusted prior to gel electrophoresis.

Samples were resolved on 0.5% agarose/TAE gels at 1.0 V/cm for 48 hours (pRS316 and pS14-8) at room temperature, or at 0.8 V/cm for 24 hours, followed by 2.2 V/cm for a further 24 hours (RCIII). Southern transfer was carried out using capillary blotting onto a positively charged nylon membrane (Hybond-N^+, GE Healthcare) as per (Southern, 1975). The membrane was probed with random-prime ^{32}P-labeled probes against the Amp^R gene (pS14-8 and pRS316) or against *LEU2* (RCIII). The blots were exposed to PhosphorImager screens (GE Healthcare) that were scanned using a Storm 860 Molecular Imager. Band intensities were analyzed and quantified using linear background subtraction and automatic band detection in ImageQuant.

Enzymes used to verify the nature of the observed bands were XhoI and PmlI (New England Biolabs), calf thymus topo I (Invitrogen), human recombinant topo I and human topo IIa (both Topogen). When statistical analyses have been performed, these have been done via paired *t*-tests using Prism software.

2.4.1 Catenation assay minichromosomes

The centromeric plasmid pRS316 (Sikorski and Hieter, 1989) and pS14-8 (containing a genomic region surrounding *RAD5* in the centromeric plasmid YCp70 (Aguilera and Klein, 1990), a gift from A.-M. Farcas and K. Nasmyth) were introduced into yeast using standard lithium acetate transformation. The ring chromosome III (RCIII) contains 61 kb surrounding centromere III, including ARS307, 308 and 309 as well as the wild type *LEU2* gene (Dershowitz and Newlon, 1993). To identify RCIII by Southern blotting, the *leu2-3.112* gene on the authentic chromosome III in the host strain was replaced with a kan^R marker (Wach et al., 1994).

2.4.2 Zymolyation of yeast cells in agarose plugs and Pulse Field Gel Electrophoresis (PFGE)

Collect approximately 10^8 cells and fix in 70% ethanol overnight at -20°C. The following day, prepare the agarose plugs by mixing 1 ml of SEZ buffer (1M sorbitol, 50 mM EDTA pH 8) with 0.02 g of LOM (low melting point) agarose and heat to 65°C to melt the agarose. Cells are centrifuged for 1 minute at 13,000 rpm and the 70% ethanol is aspirated off. Cells are then washed once in 1 ml of resuspension buffer (1M Tris pH 7.5, 1.2M sorbitol, 0.5M EDTA) before cells are resuspended in 50 µl SEMZ buffer (1M sorbitol, 50 mM EDTA pH 8, 28mM mercaptoethanol, 3 mg/ml Zymolyase 100T (MP Biomedicals)). Cells in the SEMZ buffer are then placed on a shaking heat block set at 37°C for 1 hour. After this time, the cells are mixed with the melted LOM agarose (with 3 mg/ml Zymolyase 100T added) and pipetted into plug moulds for cooling and setting. Once plugs are set, they are then incubated for 1 hour in a 37°C shaking heat block in EST buffer (10 mM Tris pH 8, 100 mM EDTA pH 8, 1% sarcosyl). After an hour, the EST buffer

is removed and replaced with 50 mM Tris pH 7.8 and 10mg/ml RNAse A and left at 37°C overnight. The following day the buffer is removed and replaced with ESP buffer (1% SDS, 1 mg/ml proteinase K, 0.5M EDTA pH 8) and incubated at 50°C for 2 hours. Finally the buffer is changed to 1X TE and plugs can be stored at 4°C. Prior to loading, plugs should be washed 3 times for 20 minutes each with 0.5X TBE or 1X TAE buffer depending on the electrophoresis protocol being used. For the last 20 minutes prior to loading, the plugs should be kept on ice.

Plugs are then loaded into 0.5% agarose gels and resolved with via PFGE. In our protocol, we used 1X TAE as the running buffer. PFGE was performed on a BioRad CHEF DR-III system at 14°C using the following program: Step 1: 24 hours at 2 V/cm, 96° angle, 1200 seconds switch time; Step 2: 24 hours at 2 V/cm, 100° angle, 1500 seconds switch time; Step 3: 24 hours at 2 V/cm, 106° angle, 1800 switch time.

2.5 Cell biology and microscopy

2.5.1 Cell cycle analysis using flow cytometry

To determine cell cycle progression by DNA content, 1 ml of culture was pelleted and fixed in 70% ethanol for at least 2 hours at 4°C. Then cells are resuspended in 50 mM NaCitrate containing 0.1 mg/ml RNAse A and put on a shaking heat block at 37°C for an hour. After the hour has passed, proteinase K is added to a final concentration of 0.1 mg/ml and the mixture left on the shaking heat block for a further hour. Cells are then pelleted and then resuspended in 50 mM NaCitrate containing 50 µg/ml propidium iodide. Cells are sonicated (Sanyo, Soniprep 150) to eliminate clumping and then are analysed on a FACScan (Becton Dickinson), with final data and graph generation prepared with CellQuest software.

2.5.2 Bi-nucleate cell counting

The fraction of binucleate cells, an indication for progression through mitosis, was scored using propidium iodide-stained cells. Scoring was repeated three times over and an average taken.

2.5.3 In situ immunofluorescence

2 ml culture aliquots are collected with an OD600 of at least 0.2. The cells are spun down and resuspended in 1 ml of ice cold formaldehyde buffer (100 mM KPO_4 pH 6.4, 0.5 mM $MgCl_2$, 3.7% formaldehyde). Cells are fixed overnight in this buffer at 4°C or can be process immediately by fixing for 2 hours at 30°C. Once fixed, cells are washed once in 100 mM KPO_4 pH 6.4, 0.5 mM $MgCl_2$, then washed once with 1 ml of spheroplasting buffer (100 mM KPO_4 pH 6.4, 0.5 mM $MgCl_2$, 1.2M sorbitol). After washing, cells are resuspended in 200 µl of spheroplasting buffer plus 2 µl of β-mercaptoethanol and 2 µl of 20 mg/ml Zymolyase 100T (MP Biomedicals) and incubated for 30 minutes at 30°C. Then cells are spun down for 2 minutes at 4,000 rpm and the supernatant is aspirated. Cells are washed with 0.5 ml of spheroplasting buffer before final resuspension in 0.2 ml of the same buffer. 5 µl of cells are then pipetted onto a polylysine coated 15 multi-well slide (MP Biomedicals). Slides are washed for 3 minutes in methanol and fixed for 10 seconds in acetone before being blocked with blocking buffer (0.5% Bovine Serum Albumin). Slides were then incubated with the relevant primary and secondary antibodies in a humid chamber in the dark for an hour each. In between incubation with each antibody, the slide wells were washed three times with blocking buffer and four times before addition of a mounting anti-fade media with 0.1 µg/ml DAPI. The antibody we used was α-tubulin clone YOL1/34 (Serotec).

2.6 Protein purifications

2.6.1 Purification of *S. cerevisiae* topoisomerase II

Overexpression and purification of *S. cerevisiae* topoisomerase II followed a published procedure (Worland and Wang, 1989). 6 hours after induction of topo II expression by galactose addition, 0.5 grams of cells were collected, washed in water, then resuspended in 200 µl lysis buffer (50 mM HEPES/KOH, pH 8.0, 150 mM KCl, 1 mM EDTA, 10% glycerol, 5 mM b-mercaptoethanol, 1 mM PMSF, and an additional protease inhibitor cocktail (Complete™, Roche). Cells were broken with acid washed glass beads to obtain greater than 90% cell lysis and the lysate collected. A 1 ml phospho-cellulose column (Whatman P11, prepared according to

the manufacturer's instructions) was equilibrated in lysis buffer. The cell lysate was loaded in batch, then the resin was packed into a column and washed by gravity flow using lysis buffer containing 200 mM and subsequently 400 mM KCl. Topo II was eluted in lysis buffer containing 800 mM KCl. This fraction was diluted back to 200 mM KCl and bound to 300 ml Q-Sepharose beads (GE Healthcare). These were then packed into a column and extensively washed with the loading buffer. A step gradient with 1M KCl was used for elution. Topo II-containing fractions were adjusted to 300 mM KCl and 10% glycerol for storage.

2.6.2 Purification of *S. cerevisiae* condensin

The 5-subunit condensin complex was overexpressed in *S. cerevisiae* as previously described (St-Pierre et al., 2009). Cell extract containing 40 mg protein in 6 ml of lysis buffer (20 mM HEPES/KOH pH 7.5, 150 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 10% glycerol, and protease inhibitors) was cleared by ultracentrifugation. The supernatant was loaded onto 200 ml of an α-HA affinity matrix (Roche). After binding, the resin was washed extensively in same buffer. Condensin was eluted by incubation with 500 µl 1.6 mg/ml HA peptide in lysis buffer over night at 4°C.

2.6.3 Interaction analysis between condensin and topoisomerase II

To carry out the interaction analysis of condensin with topo II, 25 units/ml benzonase (Sigma) was added to the cell extract containing overexpressed condensin. After ultracentrifugation, 400 µl aliquots of the extract were precleared with 20 µl of IgG-coupled Dynabeads (Invitrogen) for 30 minutes at 4°C. The beads were removed and 4 mg α-HA antibody (clone 16B12) was added to the supernatant for 30 minutes at 4°C. Then, 20 µl of protein A-coupled Dynabeads (Invitrogen) were added for a further 30 minutes. Finally, condensin-bound or control beads were incubated in the presence of 400 ng purified *S. cerevisiae* topo II for 30 minutes at 4°C. After washing, bound protein was elution in SDS-PAGE loading buffer.

2.7 In vitro assay techniques

2.7.1 kDNA decatenation assays

To assess their decatenation activity, 10 ng or 100 ng of purified yeast topo II, or 0.1 units of *E. coli* topo IV and human topo II (Topogen), were incubated with 100 ng of kinetoplast DNA (Topogen) in a 20 ml reaction containing 50 mM Tris/HCl pH 7.8, 150 mM potassium acetate, 6 mM magnesium acetate, 5 mM b-mercaptoethanol, 0.1 mg/ml BSA and 2 mM ATP. To assay the effect of condensin, reactions were incubated with the indicated amounts of condensin for 30 minutes at 37°C in the absence of topoisomerase. Then topoisomerase was added and the reaction incubated at 37°C for further 10 minutes. Reactions were stopped by addition of an equal volume of stop buffer (2% SDS, 80 mM EDTA, 600 mM NaCl) and the samples resolved on a 1% agarose/TAE gel. Gels were stained with ethidium bromide and band intensities were quantified using ImageQuant software.

Chapter 3. Results: Catenane Behaviour and Resolution

3.1 Development of the catenation assay

As discussed in the introduction, there are two aspects to chromosome resolution, the cleavage of proteinaceous links and the resolution of topological links between sister chromatids. It is only once both of these are complete that the mitotic spindle can successfully pull apart the sister chromatids to the opposite poles. Therefore in order to study the formation and resolution of sister chromatid catenanes during the cell cycle, we employed circular yeast minichromosomes of various sizes in a catenation assay that we tailored to our needs. The assay was based on a similar protocol originally used to study whether the cohesin ring physically entraps DNA, holding it together prior to seperase-cleavage and anaphase onset (Ivanov and Nasmyth, 2007). The assay is a powerful tool for tracking topological links through the cell cycle, because the minichromosomes, which unlike linear DNA, maintain any topological links once purified. Moreover, it has long been observed that larger genomes behave almost exactly like circular DNA because of the existence of domains whose fixed ends act by preventing any free rotation (Worcel and Burgi, 1972). However, in order to most closely model the endogenous chromosomes, we chose to use minichromosomes for our assay that retained many features of natural yeast chromosomes, including centromeres, replication origins and actively expressed yeast chromosome arm sequences. Thus, the minichromosomes would replicate and segregate with the same timing as the native chromosomes, mimicking their behaviour.

The first substrate we chose to use in our assay was pS14-8, a 21.2 kb centromeric mini-chromosome that contained a section of chromosome XII arm sequence around the *RAD5* gene (Figure 3.1). We chose to first use such a large substrate based on prior reports that smaller plasmids are too quickly resolved of their topological linkages by topoisomerase II to be observed in a normal timecourse (Baxter et al., 2011), thus maximising our chances of observing catenanes. For full details on the final protocol that we used, please see chapter 2.4 of the materials and methods. However, unless otherwise detailed, our

timecourses all adhered to the same general following framework: our yeast strain of interest was transformed with our assay minichromosome and cultures grown overnight. After dilution to the same optical density (OD) the following morning, cultures were arrested in G_1 through addition of the relevant mating pheromone. Once arrested, cells were washed and released into pheromone-free media that would either be shifted to 37°C or contain rapamycin (depending on the method of protein inactivation). Cells would progress through the cell cycle before re-arrest back in G_1. From the moment cells are released into pheromone-free media, aliquots for DNA purification are taken every 20 minutes as well as aliquots for FACS analysis. After the timecourse is complete, the DNA is purified from the cells and resolved on an ethidium bromide free gel at low voltage. To visualise the minichromosome we performed a Southern blot and probed against a minichromosome-specific gene. The end result is a blot showing several bands, each representing a different topological isoform of the minichromosome in question.

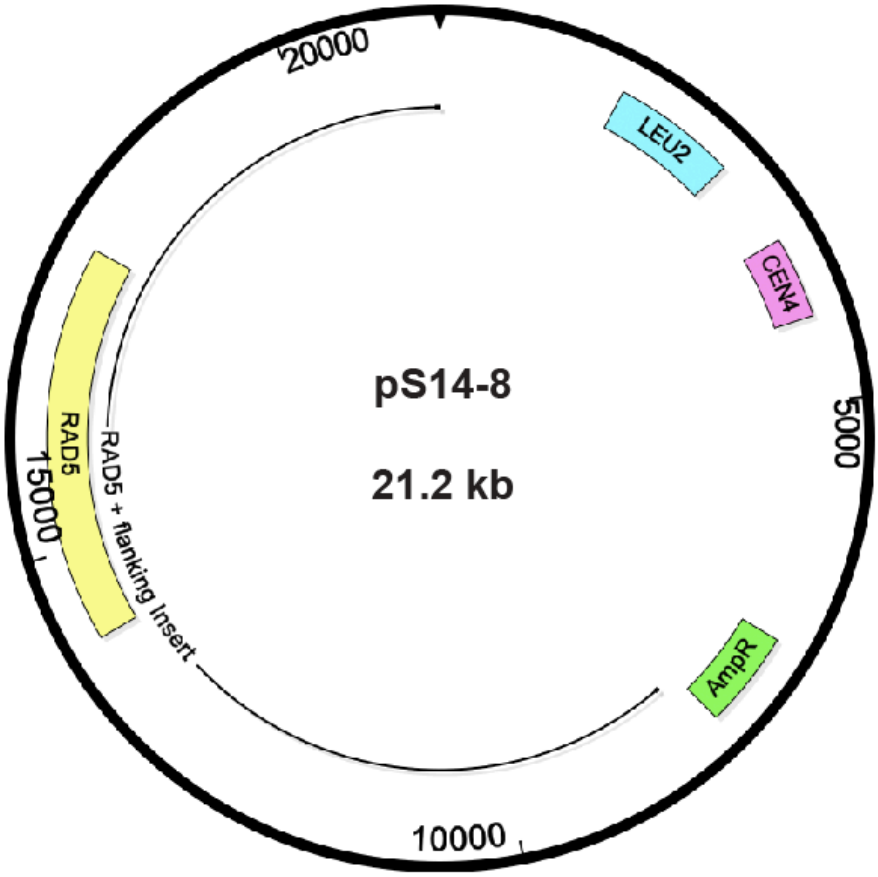

Figure 3.1 Map of minichromosome pS14-8

3.1.1 Optimisation of the catenation assay

Optimising the assay was a difficult process owing to the size of the plasmid. Size is an issue because as the plasmid size increases, its electrophoretic mobility decreases and this applies to all of its topological forms. This means that when running the DNA on the agarose gel, it can be difficult to cleanly segregate different isoforms into distinct bands, as they can all run with a similar speed. Initial blots were difficult to interpret owing to how close bands ran together, making it difficult to differentiate between them. In Figure 3.2, the first three lanes contain the purified DNA from a wild type strain containing pS14-8 (Y2665). In comparison to DNA purified from a wild type strain without pS14-8, we could clearly see some bands specific to the minichromosome, yet with poor clarity. By careful troubleshooting of the running conditions, such as the percentage of agarose used and the voltage applied to the gel, a set of conditions was reached that allowed for improved segregation of the minichromosome's topological isoforms. We found that by using a very low percentage agarose gel (0.5%) and running the samples very slowly over a long period of time (1.0V/cm for 48hrs), blots were produced that were generated greater spread of isoforms allowing for easier identification (Figure 3.3).

However looking at the blot, while the bands do segregate more clearly, there persisted another problem. Despite the specificity of the probe for the Amp^R gene, it became apparent that the first probe we had produced was also weakly labelling the genomic DNA. This can be seen as a large smeary band in the control lane where DNA was prepared from wild type cells (K699) that contained no plasmid or Amp^R gene. Unspecific labelling of the genomic DNA was further confirmed by staining gels after running with ethidium bromide to observe the genomic band. Indeed, the distance travelled by this band and the distance travelled by the smeary band on our Southern strongly suggested improper labelling of the genomic DNA by our probe. Therefore in order to remove this background labelling, we began testing a set of three new probes (labelled A, B and C) that we designed against the Amp^R gene that varied in length (Figure 3.4).

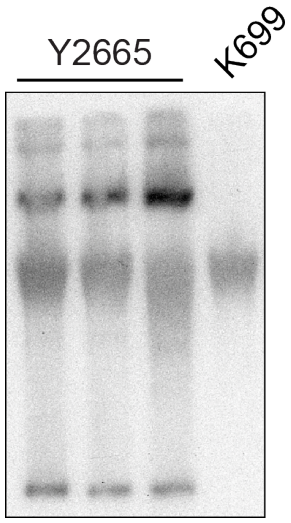

Figure 3.2 Early electrophoresis conditions showed poor isoform resolution

DNA purified from wild type cells containing pS14-8 (Y2665) is resolved electrophoretically and detected via Southern blot. Initially, running conditions were 5 V/cm for 3 hours on a 0.75% agarose gel.

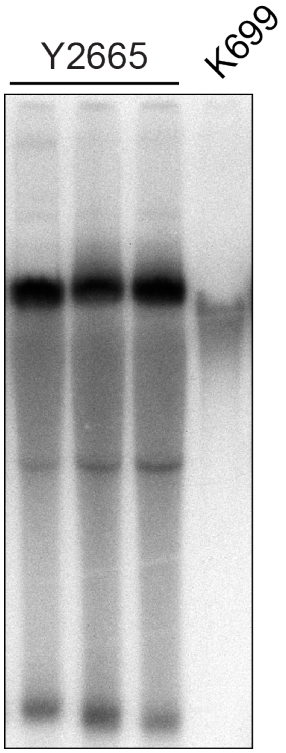

Figure 3.3 Background labelling of genomic DNA

By running samples for longer and at lower voltages (1.0 V/cm for 48 hours on a 0.5% agarose gel), a better spread of isoforms is obtained. However, our initial probe was not fully specific against the Amp^R gene and labelled the

genomic DNA as seen in the lane where only DNA purified from wild type (K699) cell is run.

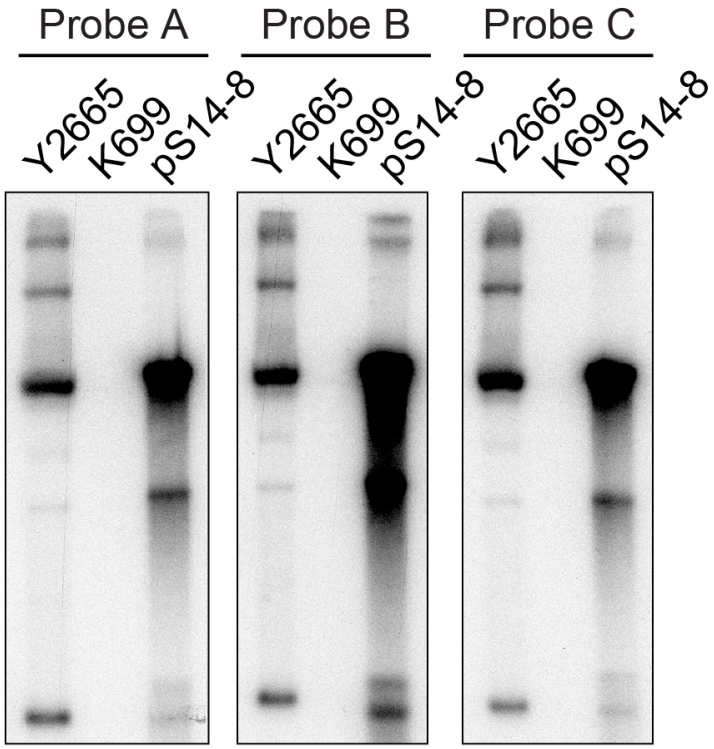

Figure 3.4 Specificity comparison of different Amp^R probes

Three new probes specific for the Amp^R gene were tested: probe A (length of probe = 469bp), probe B (524bp) and probe C (297bp). Probes were tested on DNA purified from wild type cells either containing pS14-8 (Y2665) or not containing pS14-8 (K699). Additionally, they were tested against the purified pS14-8 plasmid alone.

We used these three new probes to label either purified DNA samples from Y2665, K699 or purified pS14-8 (i.e. no genomic DNA present) to compare the intensity of their labelling as well as whether they had any non-specific labelling of the genomic. Examination of the blots showed that the new set of probes all seemed to be entirely specific for the Amp^R gene with little to no affinity for the genomic DNA, especially as when compared with our original probe as used in Figure 3.2 and 3.3. However, they did not all label the Amp^R gene with the same intensity, and we determined that probe B labelled the most clearly and strongly. As such, for all future experiments where we wished to target Amp^R, we used probe B.

3.1.2 Identifying the minichromosome's different topological isoforms

Having optimised the conditions for resolving the DNA on the agarose gel so that we could easily observe all topological isoforms, as well as having produced a highly specific probe; we could start to use the assay to study the behaviour of catenanes during mitosis. Having transformed pS14-8 into a wild type strain (Y2665), we synchronised the culture in G_1 using the relevant mating pheromone, before washing the culture and releasing the cells from their arrest. Cells were allowed to progress through one cell cycle before being rearrested in G_1. Throughout this timecourse, aliquots of cells were taken every twenty minutes and were processed to purify the DNA. This DNA was then resolved on an agarose gel at low voltage and a Southern blot performed, which was then probed against Amp^R using probe B (Figure 3.5).

Examining the blot, we can see a characteristic pattern of bands that represent the different topological isoforms of the minichromosome pS14-8. While two abundant bands are consistent throughout the cell cycle, it is clear that three new bands become apparent at the time of S-phase that then disappear again in mitosis. In order to identify which isoform each band represented, we performed a series of enzyme digests upon our purified DNA (Figure 3.6). The first enzyme used was Xho1, a restriction enzyme that only cuts pS14-8 once, hence linearizing it. Indeed from the gel, one can see that upon incubation with Xho1, all isoforms collapsed into a single linear (L) band. Linear DNA is present in all our purified DNA

preps, most likely as an unavoidable artifact of DNA shearing during the DNA purification process. The next digest performed was with topoisomerase I (topo I), an enzyme that is capable of relaxing supercoiling in DNA through the transient 'nicking' of a single strand of DNA, allowing the DNA helix to rotate and release tension (see chapter 1.5.1). By treatment with this enzyme, bands representing supercoiled isoforms of pS14-8 disappeared, with their intensity shifting to two bands likely representing their relaxed forms, relaxed monomers (RM) and relaxed catenanes (RC). The final enzyme treatment was with topoisomerase II (topo II), which differs from topo I in that it can create transient double-strand breaks, allowing the passage of one DNA strand through another, before re-ligating the DNA (see chapter 1.5.2). This means that topo II cannot only relax supercoiling (like topo I), but also can resolve topological links between different DNAs. Consequently, we saw that treatment with topo II resulted in any bands representing catenated pS14-8 disappear, leaving one intense band representing the relaxed monomer form of pS14-8. However, a smear is seen underneath this band and this likely represents partially supercoiled monomers (PSM). This is a result of topo II's bi-directionality, meaning that it can both introduce and remove supercoiling and catenation. In the lane where the DNA is treated with topo II, the smear represents monomers of pS14-8 where an equilibrium distribution of partially supercoiled monomers has been established. The reason that all the monomers are not partially supercoiled and we have a strong relaxed monomer band, is because the relaxed monomer band contains plasmids that have likely been 'nicked', i.e. had a single strand of DNA broken, either as a result of shearing during pipetting or as consequence of the enzyme treatment. If nicked, plasmids are unable to sustain any supercoiling because any tension introduced is immediately lost. Topo II can also introduce topological links, but the reason we see no catenanes is because the equilibrium is shifted very much towards decatenation. For topological links to be introduced between different plasmids, they need to be physically very close together for the reaction to occur. At their low concentration and with no proteins holding them together, it is therefore very unlikely for links to be introduced. For those plasmids that are catenated, once decatenated by topoisomerase II, the now individualised plasmids will separate spatially from each other rapidly owing to Brownian motion.

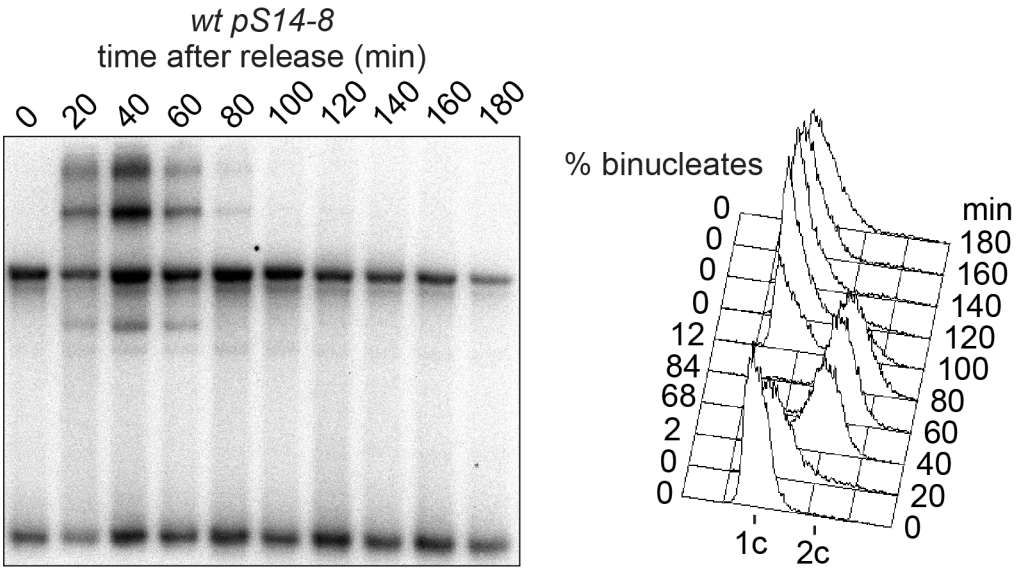

Figure 3.5 Wild type cells display a distinct isoform patterns

Wild type cells containing pS14-8 (Y2665) were synchronised in G_1 and then released, allowed to progress through one cell cycle before re-arrest. The appearance of new topological isoforms occurs when DNA replication begins, which then disappear in mitosis. FACS analysis and counting of binucleate cells confirms the synchrony of the cells and the specific timing of the appearance and resolution of these transient isoforms.

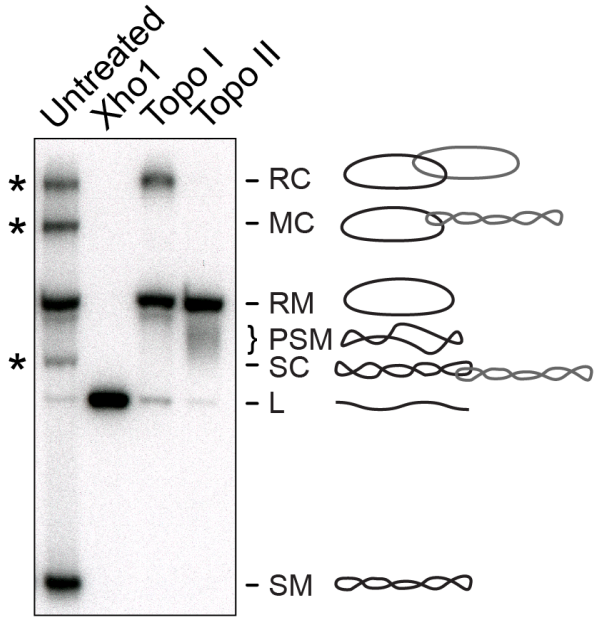

Figure 3.6 Enzyme digests allow for isoform identification

Enzyme treatment revealed the identity of isoform represented by each band; relaxed catenanes (RC), mixed catenanes (MC), relaxed monomers (RM), partially supercoiled monomers (PSM), supercoiled catenanes (SC), linear (L) and supercoiled monomers (SM). Catenated isoforms are marked with a star.

Having performed these enzyme treatments, we could confidently identify which topological isoform each band on the Southern blot represents. There are three bands detectable that represent catenated species and what is immediately striking when examining the wild type timecourse is how these catenated forms appear and then disappear with a specific pattern over the course of the cell cycle. Comparing the blot with the FACS profile, one can see that catenanes appear in S-phase when DNA replication occurs, matching the prediction made by (Sundin and Varshavsky, 1980) 30 years ago that catenanes are replication dependent. Furthermore, one can see that these catenanes are then resolved later in the cell cycle, approximately around the time of anaphase onset. We will investigate further the exact timing of this resolution in later experiments.

3.1.3 Catenation is quantifiable at each time point

To be able to observe catenanes using pS14-8, and to measure the specific timing of their appearance and resolution, was very exciting. However before investigating the behaviour of these catenanes any further, we required a way to quantify the relative amount of catenanes present at each time point taken during the time course. This would allow us to then quantify the effect certain mutants or nuclear depletions had on catenation creation, resolution and behaviour. We accomplished this through the use of ImageQuant imaging software, the final protocol for which is described in the materials and methods (chapter 2.4).

There were many ways in which to calculate the intensities of different peaks, including factors such as band detection and boundary determination. As far as possible, all band detections and subsequent calculations was performed by the program to minimise external influence on the data. However, correctly determining how to apply the subtraction of background was an area we had to optimise. Figure 3.7 shows a comparison of two methods of background deduction: the rolling ball and linear background subtraction methods. The rolling ball method is a computer determined background subtraction that ‘rolls’ an imaginary ball of set diameter along the underside of the lane profile to determine the background. However, one can see that this technique is not suited to the types of profiles generated by our data, with steep gradients seen at the start and end of the gel runs. This results in

abnormally large quantification values for the first and especially the last band. An improvement on this is the linear background subtraction along the visible background seen in the lane profile. This deducts the background evenly and fairly from each band's quantification. Indeed in Figure 3.7 we can see the differences between the two methods, which despite both quantifying the same wild-type timecourse, it is clear that the linear background subtraction method generates quantification data that best represents what can be seen on the Southern blot.

Once we had optimised the quantification protocol, we were able to start putting numbers to the observations noted in the catenation assays. Returning to our wild type timecourse (Figure 3.5), we quantified the amount of catenanes present to produce a graph showing the amount of catenanes present as a percentage of the total minichromosome in each lane (Figure 3.8). Therefore quantification reveals that during DNA replication, the percentage of catenanes peaks at around 55% before being resolved in mitosis.

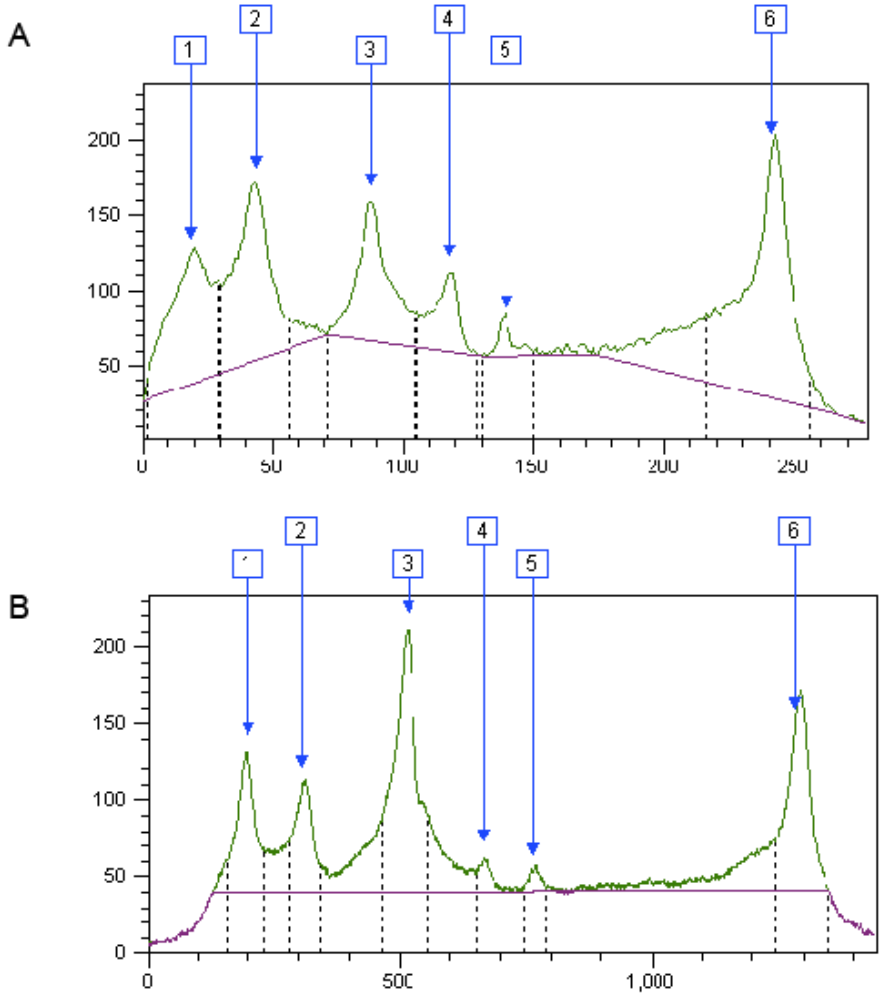

Figure 3.7 Comparison of background deduction techniques

The ImageQuant software can subtract the background via different algorithms such as (A), the 'rolling ball' method (radius = 200) or (B), a linear best fit. The above profile graphs represent individual lanes, with each number peak corresponding to a different isoform (1 = RC, 2 = MC and so on). The pink line represents the background threshold, such that the integrated values from under that line are subtracted from the final quantification values.

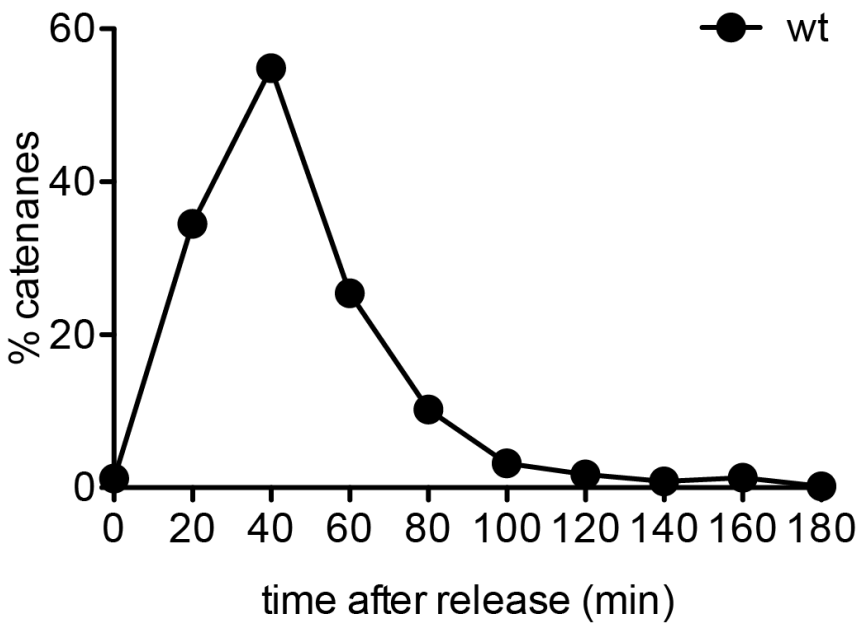

Figure 3.8 Catenane quantification of wild type cell cycle

Quantification of the Southern blot seen in Figure 3.5, where synchronous wild type cells (Y2665) were allowed to undergo one cell division. A peak of approximately 55% total catenanes was reached during DNA replication.

3.2 Spontaneous and assisted minichromosome decatenation:

With the assay established, we were keen to start examining the effects on catenation by depleting particular components of the mitotic assembly. For most of our experiments involving the inactivation of a target protein, this has been achieved through use of both temperature sensitive (*ts)* alleles and nuclear depletion via the anchor away technique (see chapter 1.7). For the relevant controls for both techniques, please see the next chapter 3.2.2.

The pS14-8 mini-chromosome harbours the efficient ARS1 early replication origin and is expected to replicate and equally segregate during every cell cycle. If catenation does indeed arise as a consequence of DNA replication, we would expect to at least transiently observe all of the mini-chromosome to be present in catenated forms. However, as seen in our wild type time course (Figure 3.5), the peak was never higher than 60%. This could be because replication does not always lead to catenation between replication products. Alternatively, it could be that all replication products are catenated, but that some sister chromatids are resolved quickly after replication, even before cells enter mitosis. To distinguish between these two possibilities, we repeated the time course analysis but inactivated the major endogenous decatenating activity of topo II using both the temperature sensitive *top2-4* mutation (Y4007) (Holm et al., 1985) and nuclear depletion via anchor away (Y4334) (Figure 3.9). With both types of depletion, we observed that upon entry into S-phase, virtually all of the minichromosomes accumulated as catenated species following DNA replication. The fact that almost no individual plasmids reappear supports the fact that without topoisomerase II, decatenation does not occur. Additionally, it was reported that without topoisomerase II, DNA replication does not progress to completion (Baxter and Diffley, 2008) an idea supported by the observation of plasmid fragmentation during anaphase as represented by the smear underneath the linear band. When comparing the *ts* mutant phenotype with the anchor away, the former appears to have a stronger phenotype. We believe that given the vast abundance of topoisomerase II present in the nucleus, anchor away may not be fully able to completely deplete the nucleus of the enzyme, or do so in a timely enough manner, while all the *ts* mutant proteins will be inactivated upon temperature shift.

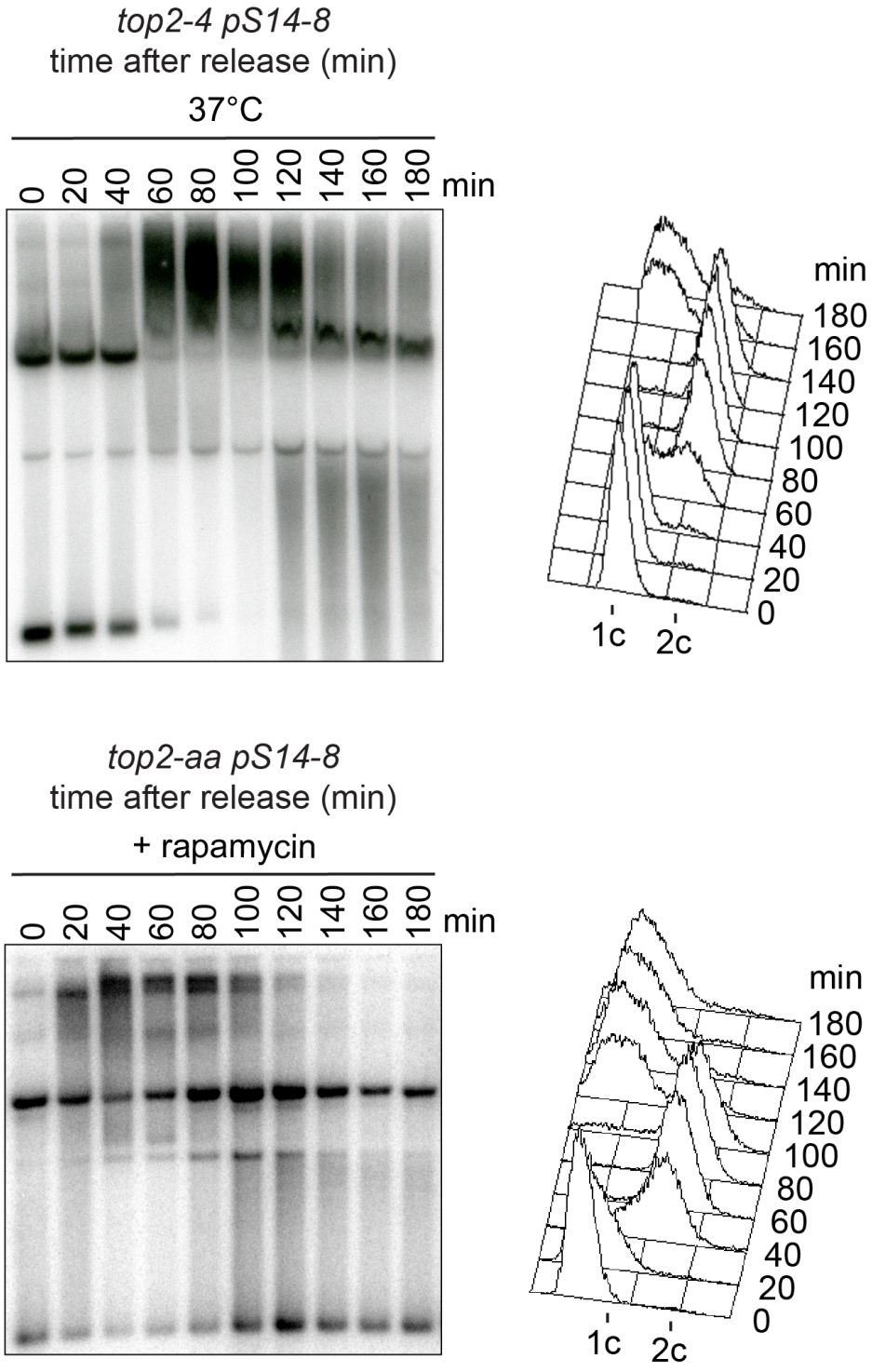

Figure 3.9 Topoisomerase II inactivation results in maximum catenane formation

Topoisomerase II is inactivated through either *ts* mutation (Y4007) or nuclear depletion via anchor away (Y4334). Both strain cultures are arrested in G_1 at

25°C before being washed and released into either media at 37°C or into media containing rapamycin.

Therefore, inactivation of topoisomerase II results in almost complete conversion of the plasmid monomers during replication to catenated forms, suggesting that S-phase results in all pS14-8 minichromosomes undergoing replication and becoming at least transiently catenated.

To estimate which portion of sister minichromosomes are resolved before progression through mitosis, we quantitatively assessed the catenation status of pS14-8 in wild type cells (Y2665) arrested in mitosis by nocodazole treatment (Figure 3.10). After a peak of catenanes during S-phase, over half of sister minichromosomes appeared to be resolved in the mitotic arrest, with approximately 40% of the minichromosomes persisting as catenanes. Thus substantial minichromosome decatenation by topo II takes place independently of progression through mitosis. We then compared a mitotic arrest by depletion of the Cdc20 co-activator of the anaphase promoting complex (Y4129) (Figure 3.11). In this strain the endogenous Cdc20 promoter has been replaced such that the addition of 2 mM methionine will stop Cdc20 expression, resulting in a metaphase arrest. In contrast to nocodazole treatment, a mitotic spindle is formed in these cells that exerts tension aimed at separating sister minichromosomes (Tanaka et al., 1999). As a result, after similar catenane levels during S-phase, only about 20% catenated molecules persisted in the arrest. This suggests that sister chromatid movement away from each other, due to tension from the mitotic spindle, promotes their decatenation.

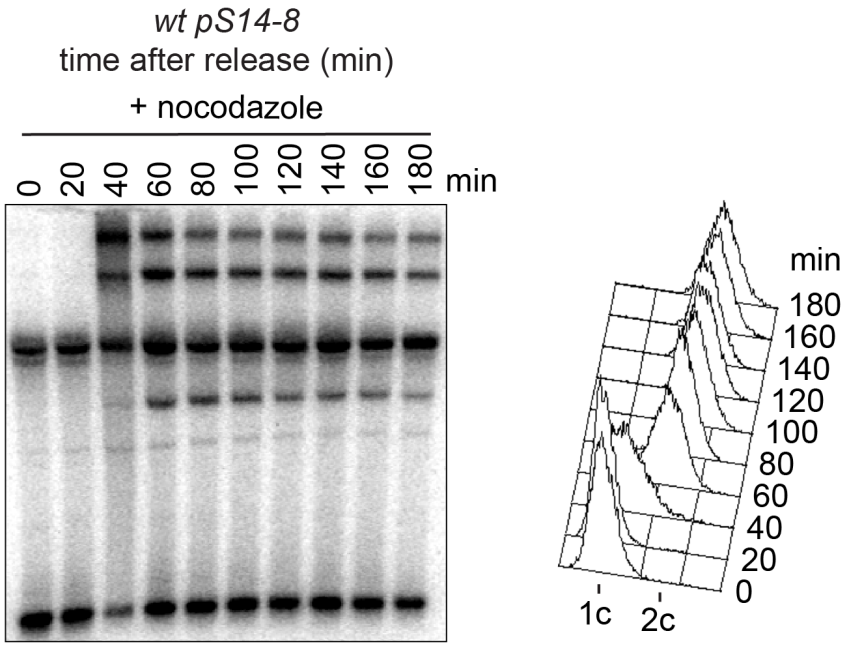

Figure 3.10 Nocodazole arrests results in catenane persistence

Wild type cells (Y2665) are released from G_1 arrest into media containing nocodazole, causing cells to arrest in metaphase. After a replication peak of catenanes, the arrest results in the majority remaining unresolved.

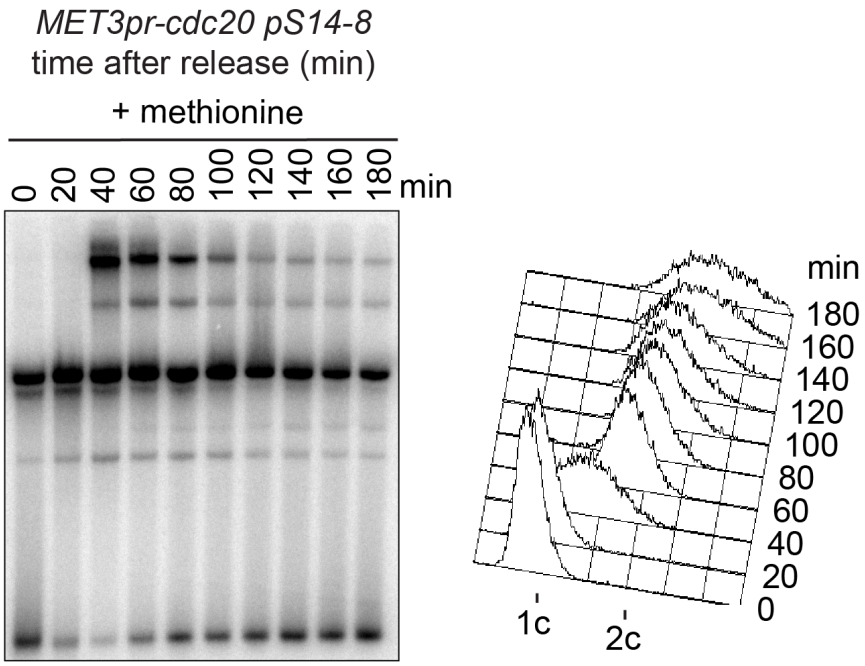

Figure 3.11 Spindle presence during arrest promotes decatenation

Like in Figure 3.10 synchronised cells are arrested in metaphase, but here via depletion of Cdc20 using strain Y4129. This results in the mitotic spindle being present, which results in fewer catenanes persisting.

Up until mitosis, movement of sister chromatids away from each other is restricted by the cohesin complex that maintains sister chromatid cohesion. We therefore addressed whether removal of cohesin changes catenane maintenance following DNA replication. After either inactivation via *ts* allele or nuclear depletion via anchor away, the abundance of catenanes during the cell cycle was markedly reduced (Figure 3.12). Even during S-phase, less than 20% of the pS14-8 minichromosomes were detected as catenated species. This suggests that sister chromatid proximity, afforded by the cohesin complex, protects catenation. In the absence of cohesin, sister chromatids move apart and are readily decatenated by topo II. The conclusion that cohesin protects sister chromatid catenation was recently reached independently (Farcas et al., 2011). Quantification of the catenanes for all the above time courses can be seen and compared in Figure 3.13.

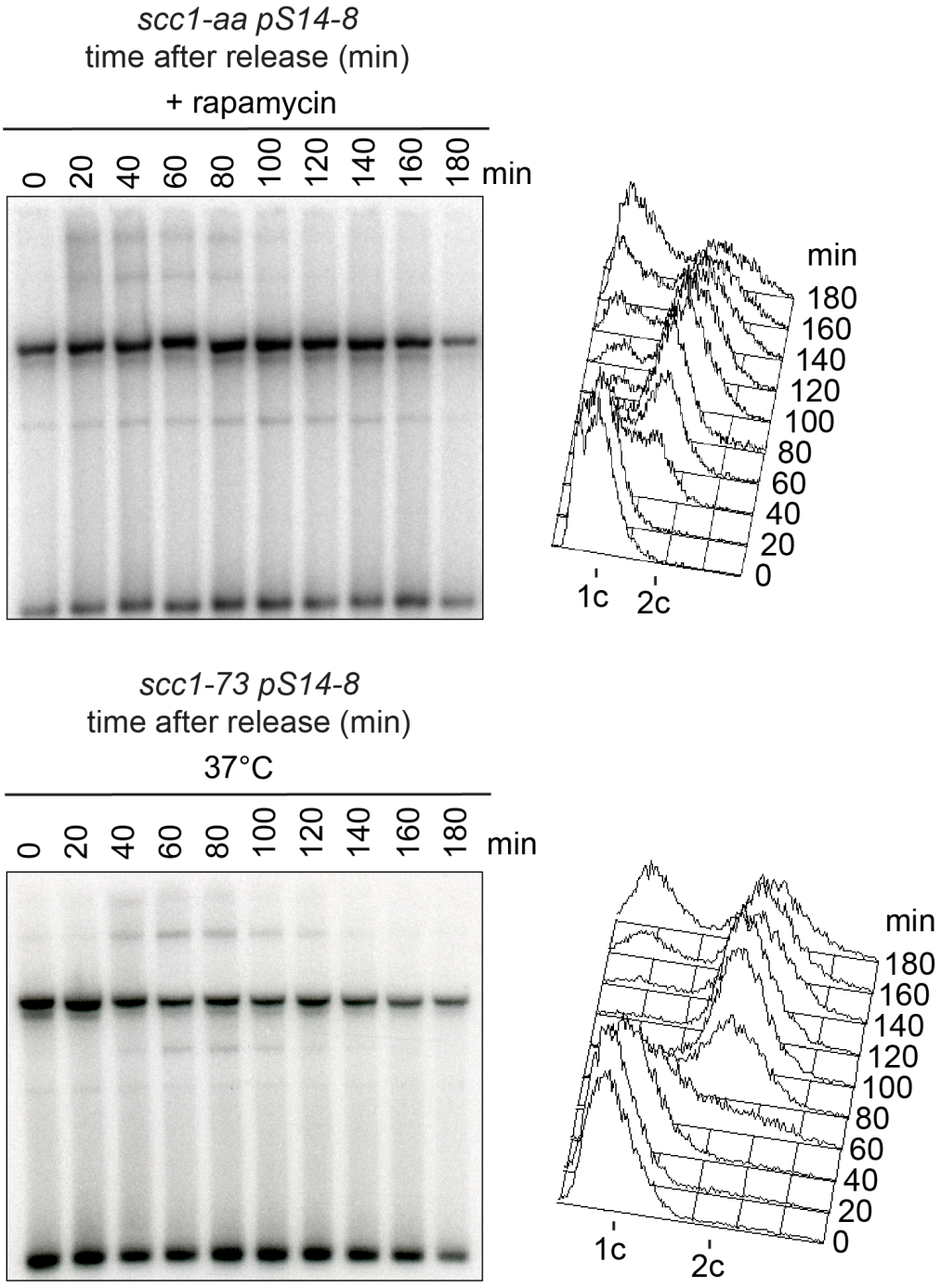

Figure 3.12 Inactivation of cohesin results in fewer observable catenanes

Cohesin is inactivated through either *ts* mutation (Y4029) or nuclear depletion via anchor away (Y4201). Both strain cultures are arrested in G_1 at 25°C before being washed and released into either media at 37°C or into media containing rapamycin.

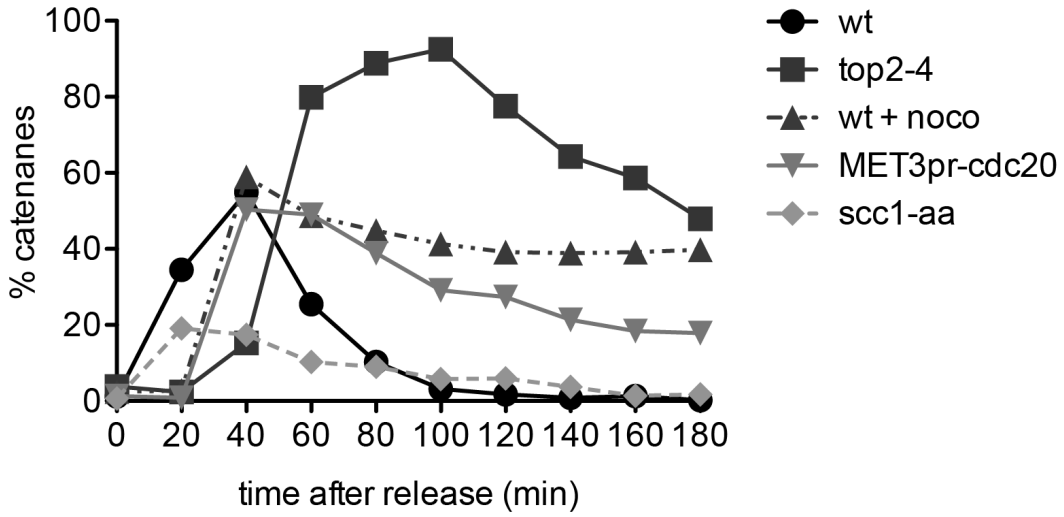

Figure 3.13 Catenane quantification for different mitotic inactivations

3.2.1 Temperature's effect on catenation and isoform distribution

In the previous chapter, we have been using two different approaches to inactivate target proteins: protein inactivation via *ts* alleles and nuclear depletion via anchor away. Both of these procedures will inactivate the target protein but do so by differing means, one requires a dramatic temperature shift, the other the addition of the compound rapamycin. Given that both techniques alter the cellular environment, we wanted to check that these depletion methods themselves did not affect our catenation assay via the appropriate control experiments.

Initially, we examined the effect of temperature on our catenation assay. To examine this, we released synchronous wild type cells (Y2665) from a G_1 arrest and allowed them to proceed through one cell cycle before re-arrest. This time course was done at both 25°C and 37°C and the Southern blots were quantified and compared (Figure 3.14). We can see that the pattern and distribution of bands and their relative intensities is altered between the two different temperatures. Moreover, a very slight amount of persistent catenation was detected in the 37°C samples suggesting that the insult of the higher temperature could have some slight effect on catenation, perhaps via impairment of topo II activity.

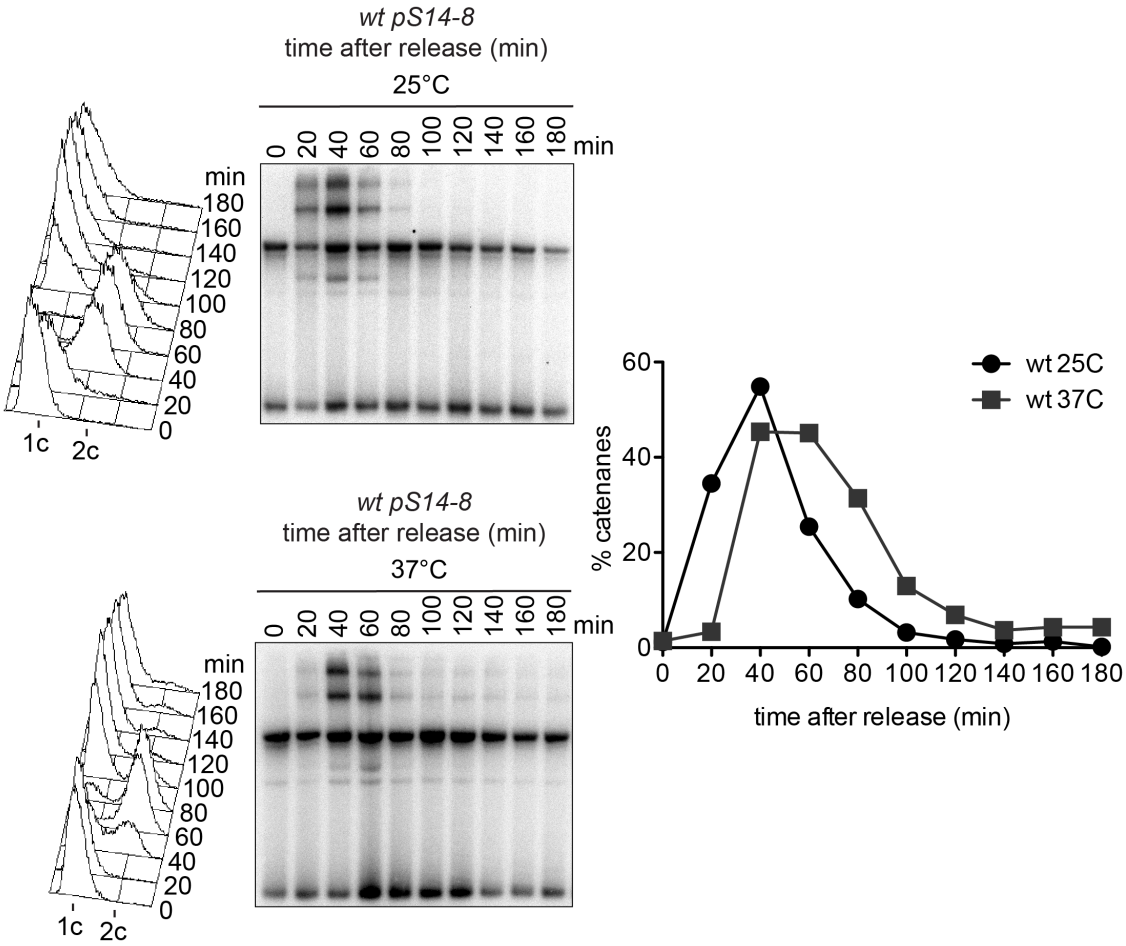

Figure 3.14 Effect of temperature on catenane behaviour

Cells (Y2665) synchronised in G_1, were released from their arrest into media either at 25°C or 37°C and allowed to progress through one cell cycle before re-arrest in G_1.

Following on from these observations, we also wanted to examine in more detail the effect of temperature on the different topological forms. In the previous results chapter 3.2, we found that a nocodazole arrest results in a catenation equilibrium being established where all the different topological forms are present and can clearly be seen. We repeated this experiment but this time performing the experiment at 37°C instead of 25°C then proceeding to quantify and compare the experiments (Figure 3.15). From this comparison, we made two interesting observations; the first was that the overall amount of persistent catenation at either temperature was very similar. The second was that the amount of supercoiled species, both monomers and catenanes, was markedly reduced at higher temperatures throughout the entire time course when compared to the levels seen at 25°C. This is an unsurprising result, considering that increasing the temperature will increase the kinetic energy of small molecules such as DNA. At higher temperatures, collisions, interactions and reactions between molecules will occur with greater energy. It is likely that this could translate to a higher occurrence of DNA strand-nicking and as discussed earlier, plasmids that contain nicked DNA are unable to sustain any supercoiling. In conclusion, these temperature comparisons have shown that while increased temperature does affect the various ratios of topoisomers present, the overall relative amount of catenation remains very similar.

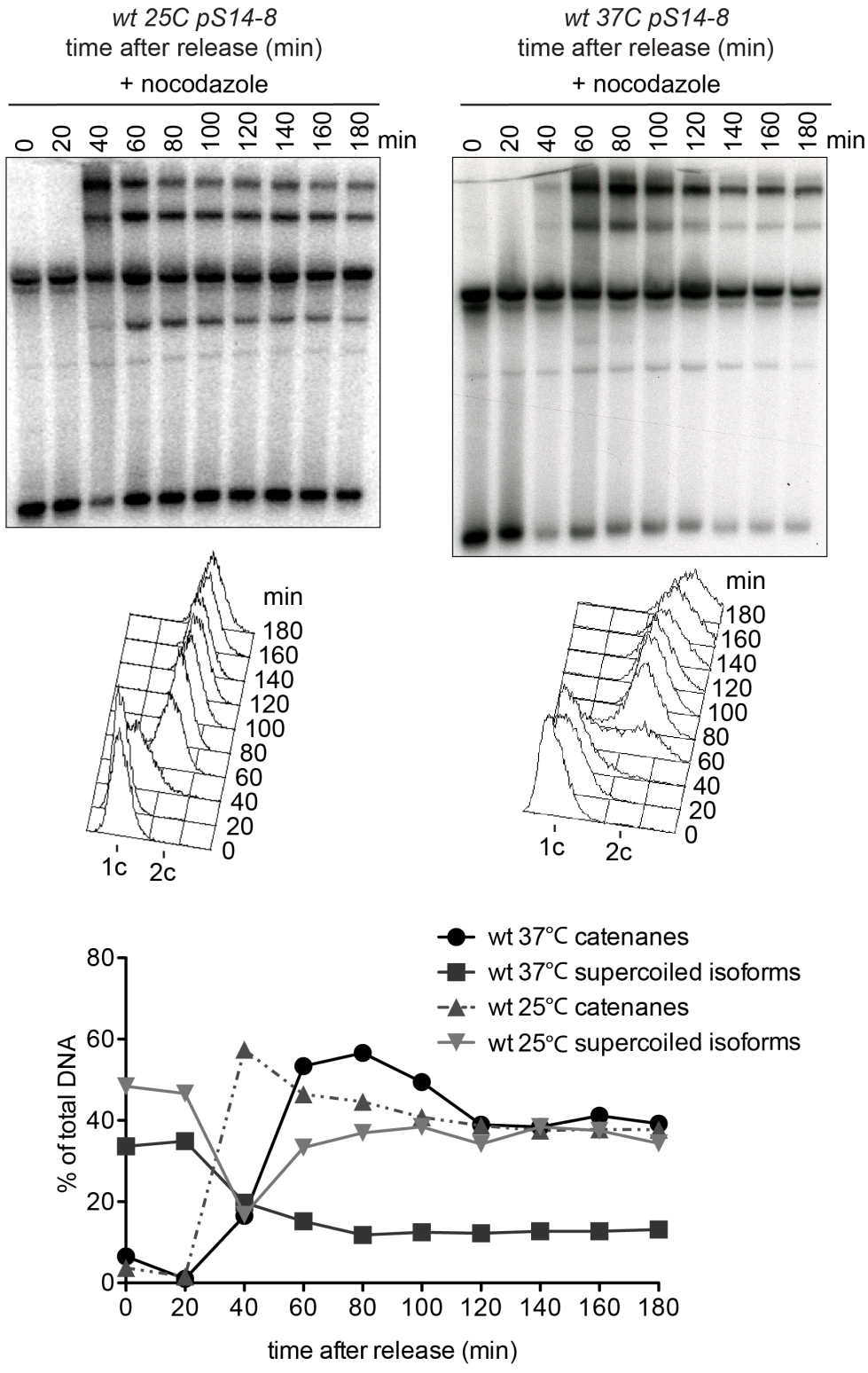

Figure 3.15 Effect of temperature on different isoforms

Wild type cells (Y2665) were synchronised in G_1 and released into a nocodazole-induced metaphase arrest at either 25°C or 37°C. Quantification revealed that while overall catenation remained similar, supercoiled isoforms (both monomers and catenanes) were reduced at the higher temperature.

3.2.2 The anchor away system does not effect catenation

When the new anchor away technique was published (Haruki et al., 2008a), our lab quickly obtained the strain and modified it for our own ends by tagging our proteins of interest with the FRB tag (see chapter 1.7 for an introduction to the anchor away technique). Having tagged our target proteins, we wanted to check that the parental strain itself, as well as the presence of rapamycin, did not exert any influence on catenane behaviour. The anchor away strain has certain mutations inherent to its design. Because rapamycin is toxic to wild-type *S. cerevisiae*, the anchor away strains are made rapamycin-resistant through mutation of *TOR1 (tor1-1)*. Additionally *FPR1 (Δfpr1)* is deleted, as it is the yeast homolog of the human FKBP12 gene that encodes the most abundant FK506 and rapamycin binding protein in *S. cerevisiae*. This is necessary to reduce competition between Fpr1p and the anchor-FKBP12 construct for binding to the FRB domain (Heitman et al., 1991). Therefore given the modifications of the strain, plus the exposure to the usually toxic rapamycin, we needed to ensure that neither had any effect on the creation and resolution of catenanes. To test this, we performed a time course using an anchor away 'parental' strain (Y4332) where no target protein has been FRB-tagged. These cells were synchronised and released into media containing rapamycin and allowed to progress through one cell cycle (Figure 3.16). As can be seen, neither the strain nor the presence of rapamycin appears to have any influence on catenation and the pattern of topological forms looks the same as with our wild-type strain (Figure 3.5).

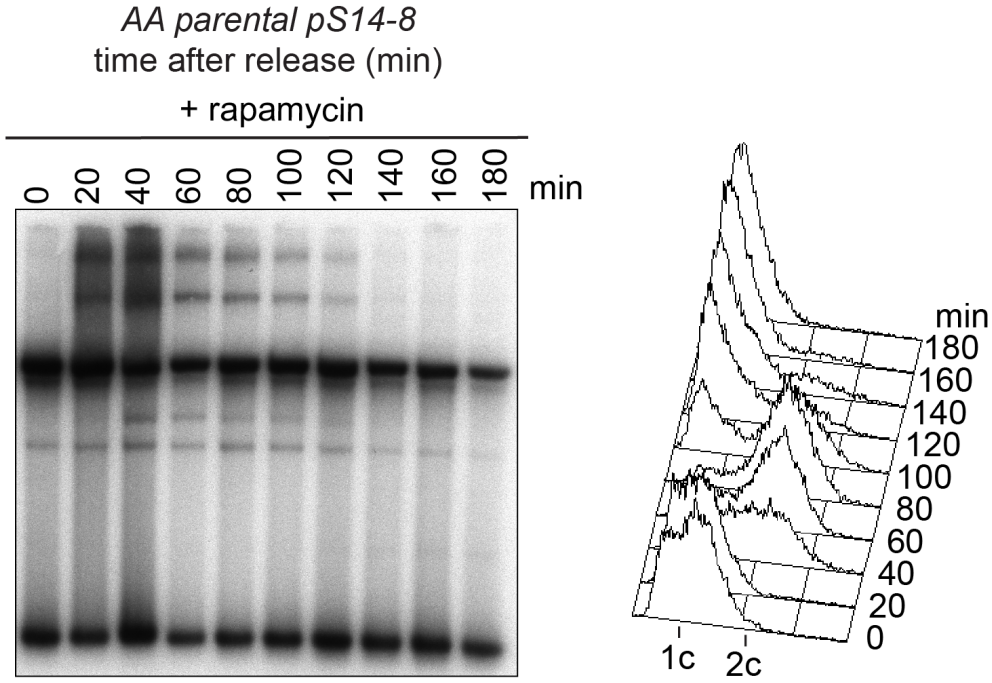

Figure 3.16 Anchor away and rapamycin do not affect catenation

Strain Y4332, which does not contain any target FRB tagged protein, was synchronised in G_1 and then released into media containing rapamycin. After progressing through one cell cycle, cells were re-arrested back in G_1.

3.3 Catenation of smaller minichromosomes is observable in wild type strains

Having established the assay using the minichromosome pS14-8, and having seen how clearly we were able to observe catenanes, we wanted to check that our results were not unique to this one particular plasmid. Previous catenation studies involving smaller minichromosomes, specifically pRS316 (4.88kb) (Figure 3.17), have reported that under wild type conditions, catenanes were resolved too quickly during mitosis to be detected and could only be observed through inactivation of endogenous topo II (Baxter et al., 2011). However, given the clarity of our results with pS14-8, we decided to test a smaller minichromosome in the assay to see if this was indeed the case. We also used pRS316 and apart from changing the electrophoresis running conditions, used the same protocol for purification and resolution of the DNA as with pS14-8. We performed a time course using WT cells (Y4199), quantified the data and were able once again to observe the appearance and then resolution of catenanes during the cell cycle (Figure 3.18). To confirm the detection of catenanes, enzyme digests were performed on the DNA samples to confirm what topological forms each band represented. Digests with topo I and II revealed that the top two bands with the lowest electrophoretic mobility indeed represented catenated forms. While the top band contains relaxed catenanes, the second catenated band most likely contains a mix of both supercoiled and mixed catenanes. While with pS14-8 these two forms ran separately, it seems most likely that with the smaller pRS316, the supercoiled and mixed catenanes have a similar electrophoretic mobility. The linear form of pRS316 was not detectable in our samples, suggesting that these smaller DNA molecules are less susceptible to shearing during purification than their larger counterparts. Comparing the results with pS14-8, it was reassuring to obtain similar results with the much smaller minichromosome and additionally confirm that our catenation assay was not particular to any one plasmid but rather representative of them all. However, we can see from the quantification that the smaller pRS316 had a peak catenation of under 30%, around half of the peak seen for pS14-8, suggesting that most of the plasmid molecules were decatenated soon after synthesis. So while it is very much possible to observe the catenanes of smaller plasmids under wild type conditions, their smaller size seems to result in faster decatenation.

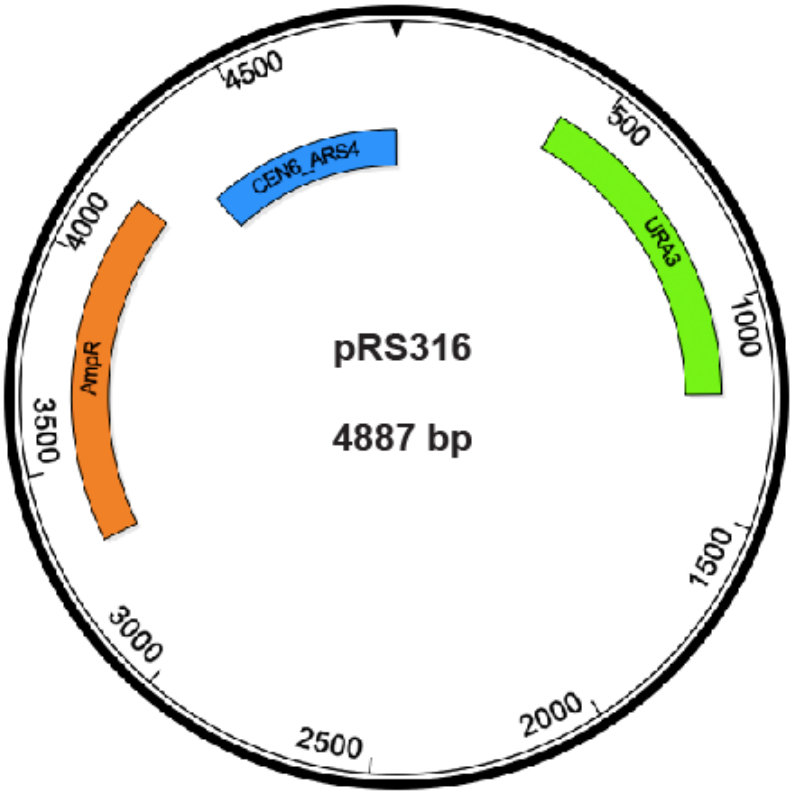

Figure 3.17 Map of minichromosome pRS316

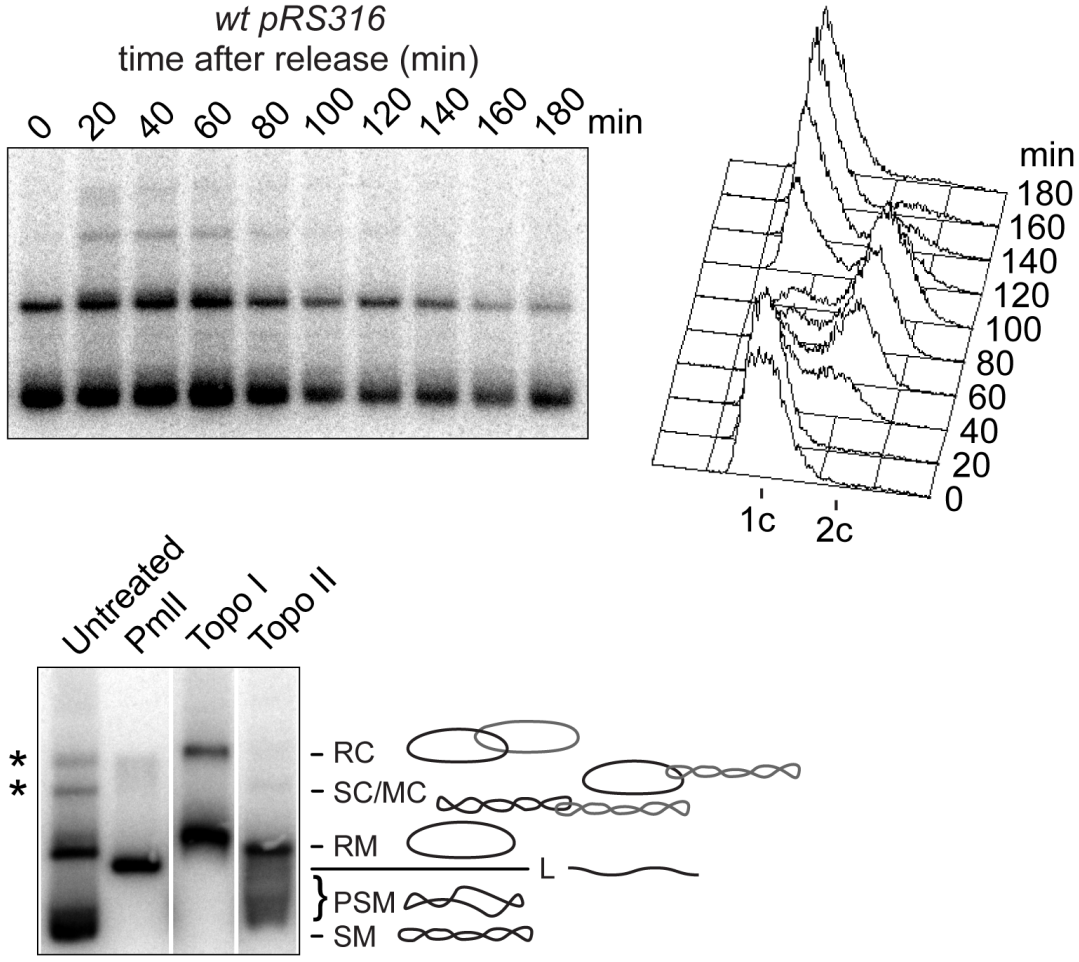

Figure 3.18 Catenation detection and isoform identification for pRS316

Catenanes can be detected in wild type cells containing pRS316 (Y4199). Cells are synchronised in G_1 before release, and are allowed to progress through one cell cycle before re-arrest in G_1. Enzyme treatments were used to determine which isoform each band represented; relaxed catenane (RC), supercoiled catenane (SC), mixed catenane (MC), relaxed monomer (RM), linear (L), partially supercoiled monomers (PSM) and supercoiled monomer (SM). Catenated isoforms are marked with a star.

Chapter 4. Results: Condensin's Influence on Catenation

4.1 Condensin promotes completion of minichromosome decatenation

Having established our catenation assay and studied some of the major mitotic components, it was time to begin studying condensin directly. The first and simplest approach to take was to inactivate condensin using one of the *ts* mutants in our laboratory's possession. Therefore, we performed timecourses with three condensin *ts* alleles: *smc4-1* (Y3954), *brn1-9* (Y3939) and *ycg1-10* (Y3940), each containing the 21 kb minichromosome, pS14-8. In all cases, cells were synchronised in G_1 at the permissive temperature before being released into media at the non-permissive temperature (Figure 4.1). Looking at the results for all three mutants and comparing them with wild type cells shifted to 37°C, one can see a subtle but constant phenotype of partial persistent catenation once mitosis is complete. Quantification of the blots reveals that after peaking at approximately 50% catenanes 60 minutes after release from G_1, the cells still retain 10-20% catenanes total 180 minutes after release. When compared to wild type cells, that have less than 5% catenanes upon re-arrest in G_1, these results clearly show that with condensin inactivated, there is a failure to completely resolve catenation. The FACS profiles for all three condensin mutants also show a characteristic double peak or 'shoulder' as the cells are re-arrested in G_1 at the non-permissive temperature. This is a characteristic condensin phenotype (D'Ambrosio et al., 2008a) attributed to chromosome mis-segregation.

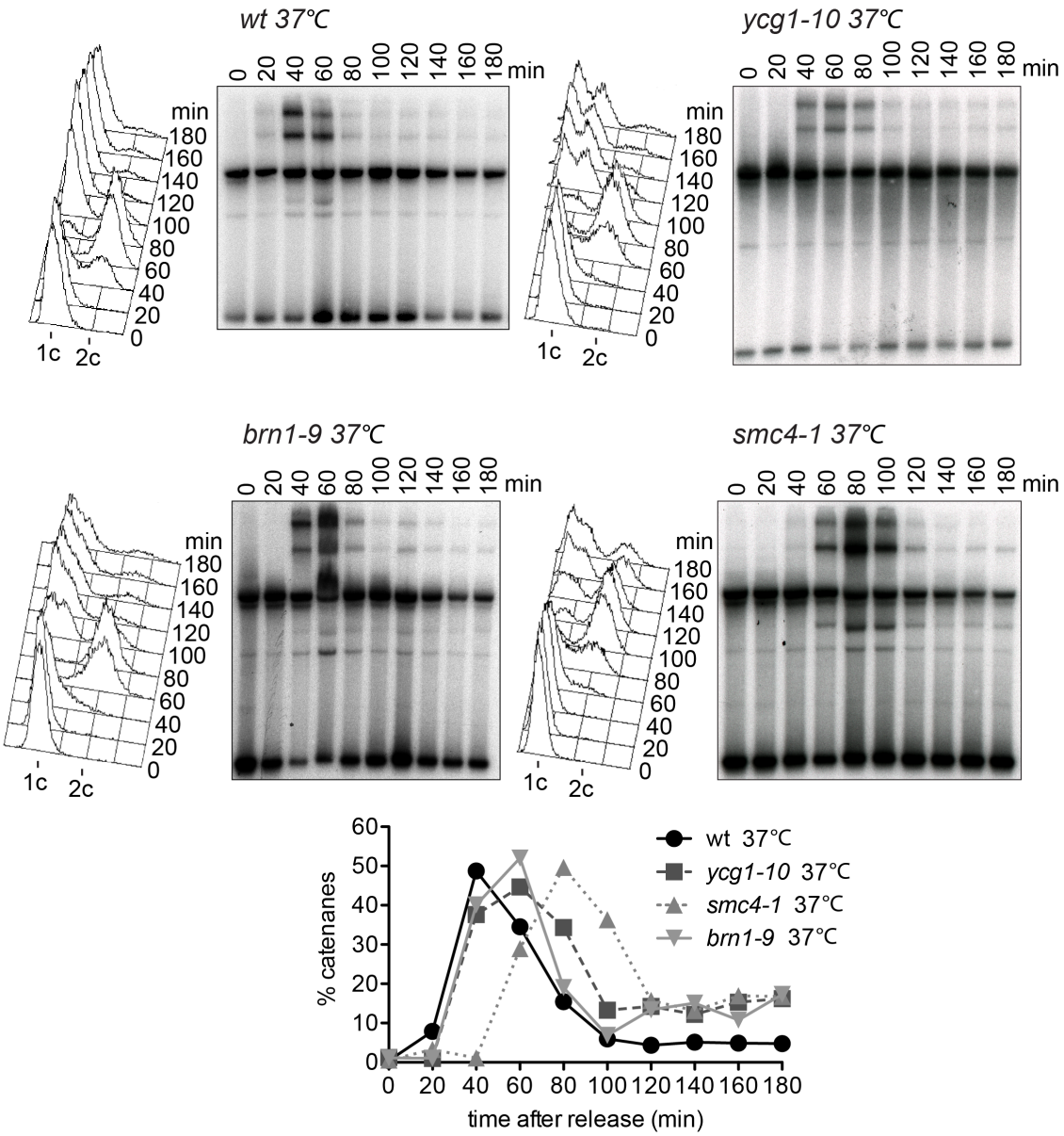

Figure 4.1 Condensin *ts* mutants all display a persistent catenane phenotype

Condensin mutants *ycg1-10* (Y3940), *brn1-9* (Y3939) and *smc4-1* (Y3940) are synchronised in G_1 before release into media at the non-permissive temperature. For all *ts* mutants, persistent unresolved catenation is detectable when compared to wild type (Y2665) cells.

Having obtained these results with the *ts* mutants, we were then very keen to see if similar results were obtained via nuclear depletion of condensin using the anchor away technique. Therefore, we analyzed catenane abundance in synchronised cells following nuclear depletion of condensin using an anchor away allele of its Brn1 subunit (*brn1-aa* (Y4059) Figure 4.2). Catenanes accumulated during S-phase to comparable levels, irrespective of whether rapamycin was added to deplete condensin. During G2 and mitosis catenanes started to be resolved under both conditions but, in contrast to wild type cells that saw complete decatenation, decatenation progressed more slowly in condensin-depleted cells and approximately 15% catenanes persisted unresolved until the end of the experiment. This was not because mitosis was delayed in the absence of condensin. FACS analysis of the DNA content, as well as the budding index, demonstrated that both cultures progressed through the cell cycle with similar kinetics and all cells had returned to G_1 by the end of the time course. Anaphase bridges and chromosome missegregation were abundant after rapamycin addition, as seen on the FACS profile and in anaphase cells stained with the DNA dye 4',6-diamidino-2-phenylindole (DAPI; Figure 4.2), verifying that the depletion of condensin was not just having some minichromosome-specific effect. Persistent minichromosome catenation after condensin depletion was reproducibly observed at similar levels in several biological repeats of this experiment (Figure 4.3). Therefore, these results suggest that condensin promotes resolution of sister chromatid catenanes and demonstrates that in the absence of condensin a fraction of catenated minichromosomes persist until the time of cell separation by cytokinesis.

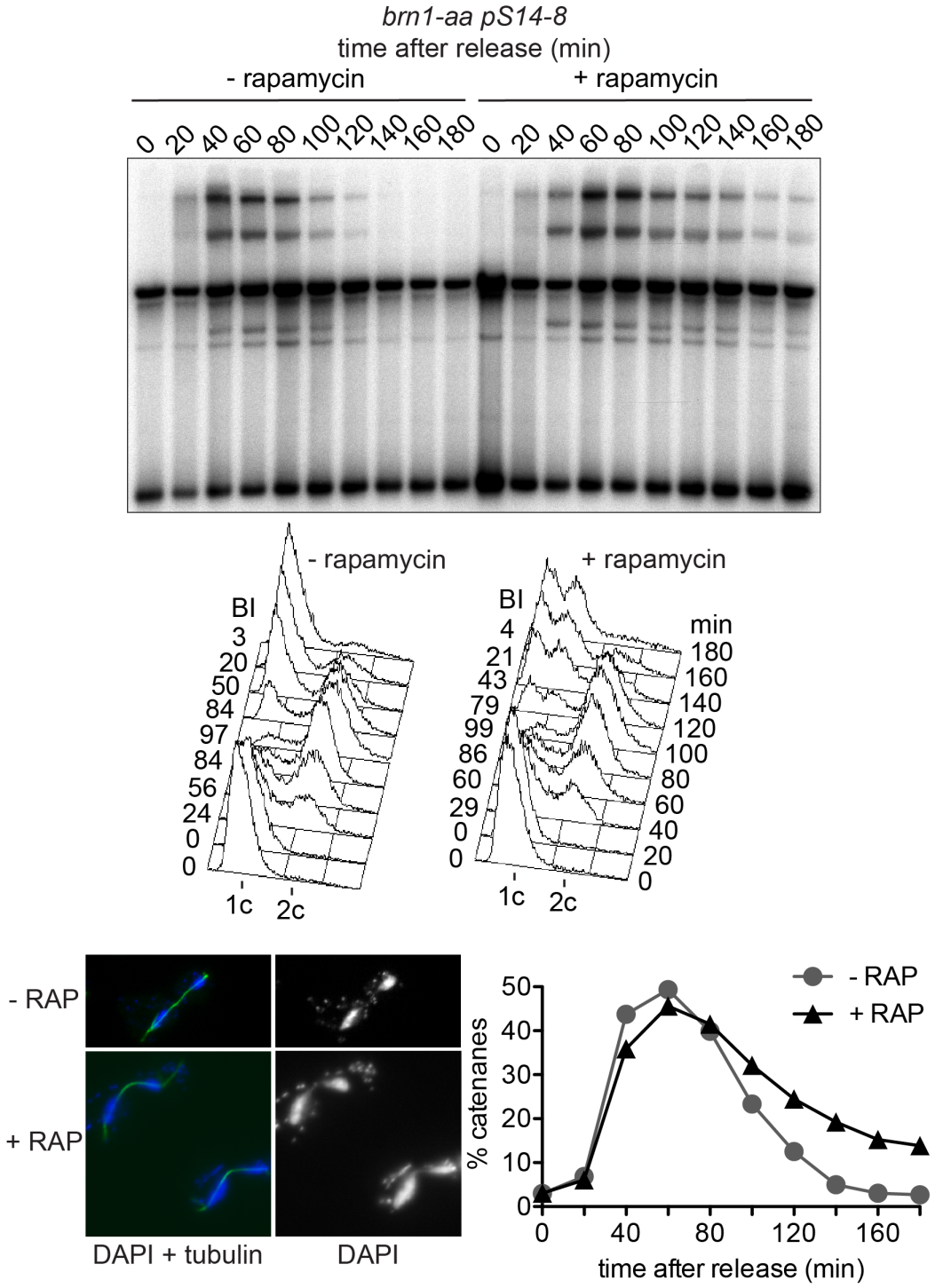

Figure 4.2 Condensin is required for complete decatenation

Condensin depletion causes part of the minichromosome catenanes to remain unresolved. Condensin was depleted via anchor away depletion of Brn1 (Y4059) before release from G_1 arrest and allowed to proceed trough one cell cycle before re-arrest. The percentage of catenanes was quantified Immunofluorescence microscopy of cells stained with a α-tubulin antibody and the DNA dye 4',6-diamidino-2-phenylindole (DAPI) reveals anaphase bridges after condensin anchor away.

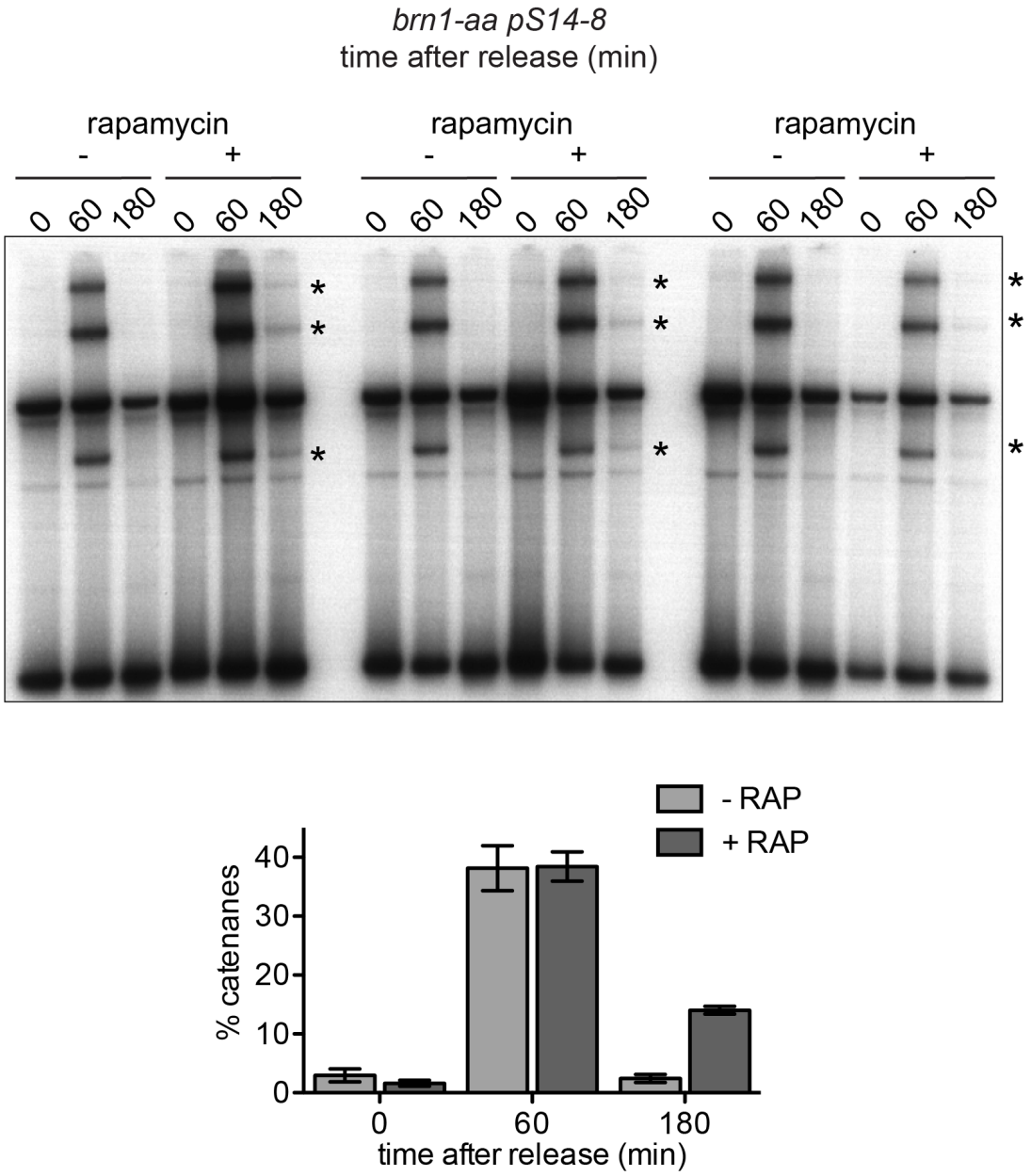

Figure 4.3 brn1-aa triplicates and quantification

The experiment shown in Figure 4.2 was repeated 3 times and the percentage of catenanes at the indicated time points quantified. Only time points at 0, 60 and 180 minutes after release were taken. The mean and standard deviation are shown. Catenanes are marked with a star.

If our circular minichromosome is a faithful model for the behavior of authentic chromosomes, then the condensin requirement for complete decatenation could explain anaphase bridges and chromosome missegregation in the absence of condensin. One caveat to this conclusion is that if condensin was required to resolve different, catenation-independent, linkages on authentic chromosomes, then such linkages might indirectly hamper minichromosome decatenation. We therefore addressed whether condensin contributes to chromosome decatenation independently of promoting chromosome movement. To do this, we arrested cells in mitosis using nocodazole, a situation where the mitotic spindle is disrupted and therefore directed chromosome movement no longer contributes to sister chromatid resolution. Cohesin was depleted from the nucleus using the *scc1-aa* allele, as above, allowing spontaneous catenane resolution (Figure 4.4). Condensin depletion in the same cells, via the *brn1-aa* allele, led to an increased population of persisting catenanes. This suggests that condensin supports chromosome decatenation independently of, or in addition to, promoting efficient chromosome movement during anaphase. However, paired *t*-test analysis comparing the two strains produces a P value of 0.0497, which only just implies a significant difference at a confidence level of 95%. Following a similar line of thinking, we wanted to confirm that condensin also supports decatenation independently of mitosis progression. In Figure 4.5, *smc2-aa MET3pr-cdc20* cells were arrested with addition of methionine either in the presence of rapamycin or not. One can see that when condensin is depleted, more catenanes persist, thus suggesting that the mechanism by which condensin promotes decatenation is active prior to the metaphase to anaphase transition. The effect of condensin here is more significant, with a paired *t*-test analysis producing a P value of 0.0010, suggesting that there is indeed a significant difference resulting from condensin depletion.

Taking these results together, we suggest that persistent catenanes in the absence of condensin are a likely source of chromosome segregation failures.

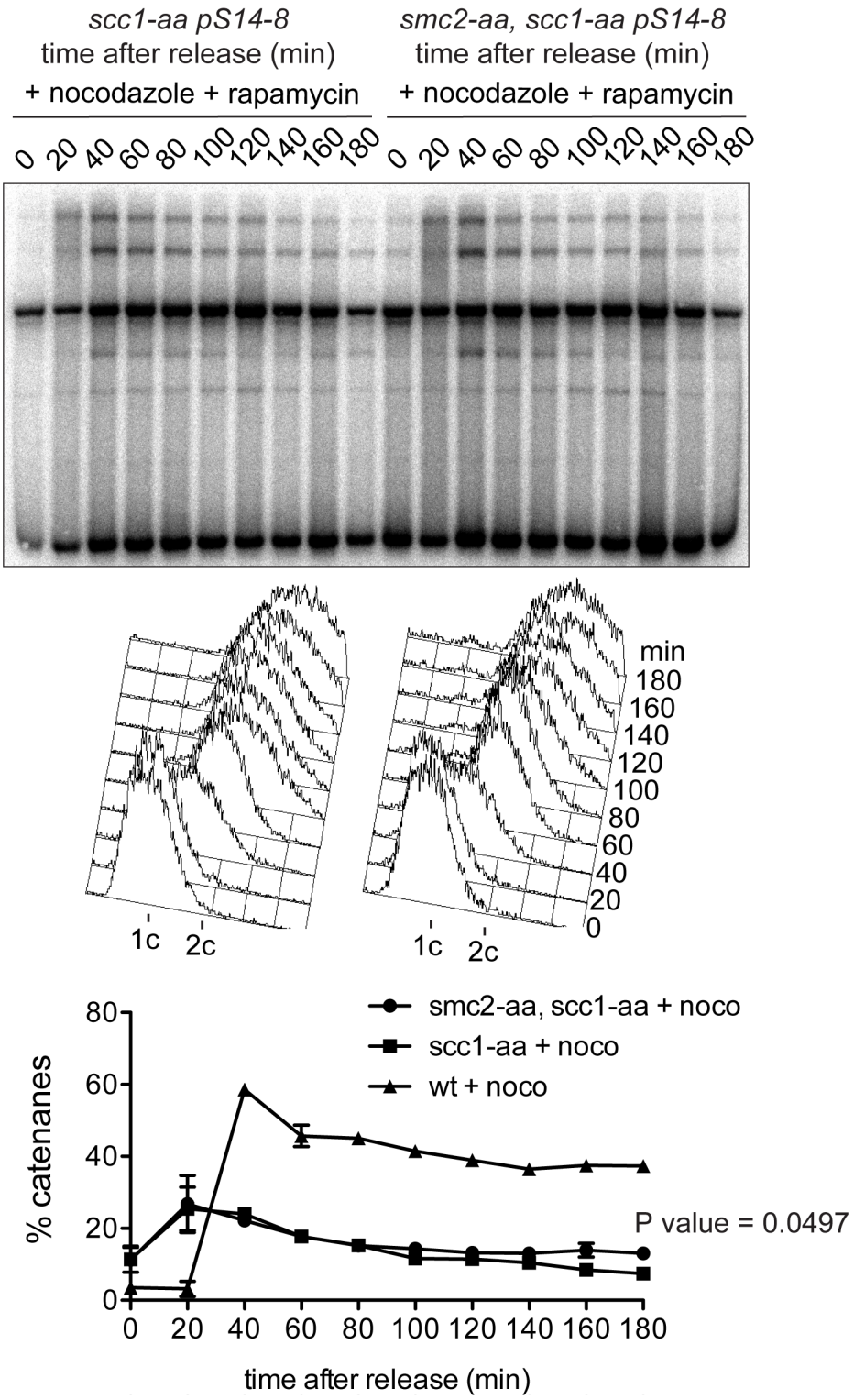

Figure 4.4 Condensin contributes to decatenation independently of chromosome movement

Cells of the indicated genotypes (Y4201, Y4235) were synchronised in G_1 and released into media containing nocodazole and rapamycin. Catenanes were quantified and plotted with a comparison to arrested nocodazole wild type cells.

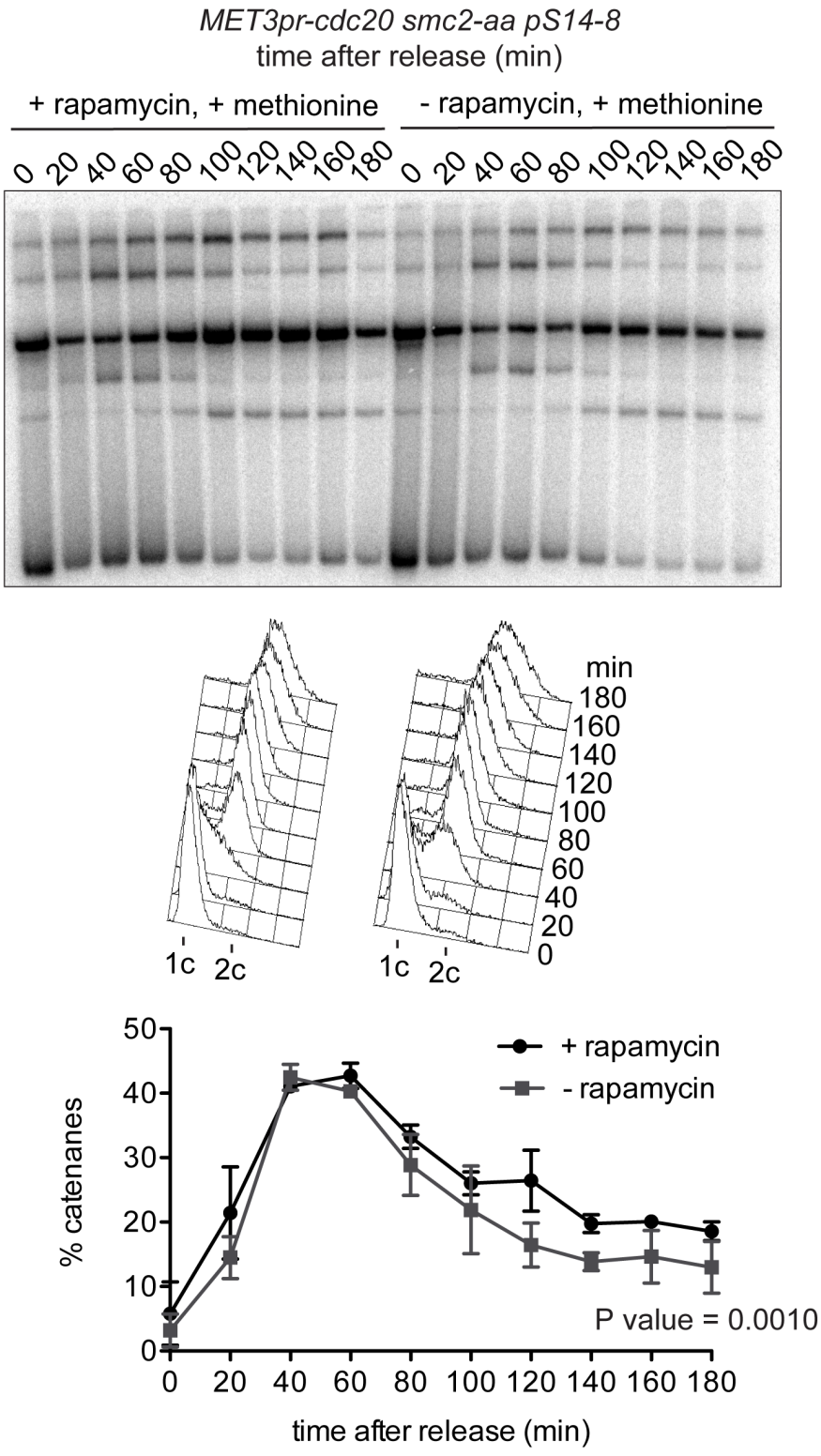

Figure 4.5 Decatenation is active prior to anaphase

Cells of the indicated genotypes (Y4113) were synchronised in G_1 and released into media containing methionine and either rapamycin or no rapamycin. Catenanes were quantified and plotted.

4.2 The effect of minichromosome size

This clear observation of persistent catenation resulting from the inactivation of condensin was stimulus to see if we observed a similar phenotype when employing the smaller pRS316 plasmid. We began by investigating the effect of the *ts* mutant *smc2-8*, in order to make a direct comparison to results from a previous study (Baxter et al., 2011) where catenanes had not been seen upon shift to the non-permissive temperature (Figure 4.6). In our timecourse we do clearly see catenanes appear and it seems that a portion of these do persist. However, this particular mutant had difficulty in release synchronously from the G_1 arrest such that we could not be certain that the catenanes observed 120 to 180 minutes after release were not a result of late comers entering S-phase. The FACS profile is particularly unclear for this strain (Y4236) making assessment of cell synchrony difficult. Fortunately, we also had the anchor away method at our disposal (Figure 4.7). Nuclear depletion of condensin revealed a small delay in catenane resolution, but the effect was less pronounced compared to the larger minichromosome. However the FACS profile does show the distinct 'shoulder' upon re-arrest in G_1 that is commonly seen in condensin mutants. By the time cells passed through mitosis in the absence of condensin, less than 10% of catenanes persisted. This suggests that condensin makes a contribution to plasmid decatenation, albeit small. We note that if condensin-dependent positive supercoiling was required for pRS316 decatenation, which has been observed in topo II depleted cells (Baxter et al., 2011), we should have observed a greater resolution defect.

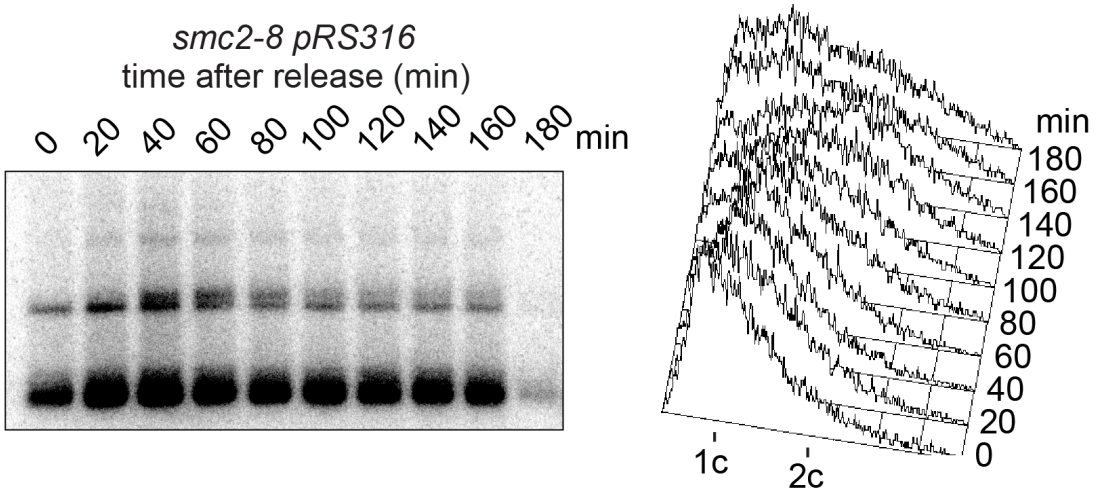

Figure 4.6 *smc2-8* releases poorly from G_1 arrest

smc2-8 cells (Y4236) arrested in G_1, washed and placed into fresh media at 37°C, release from the arrest asynchronously making the tracking of catenane appearance and resolution difficult.

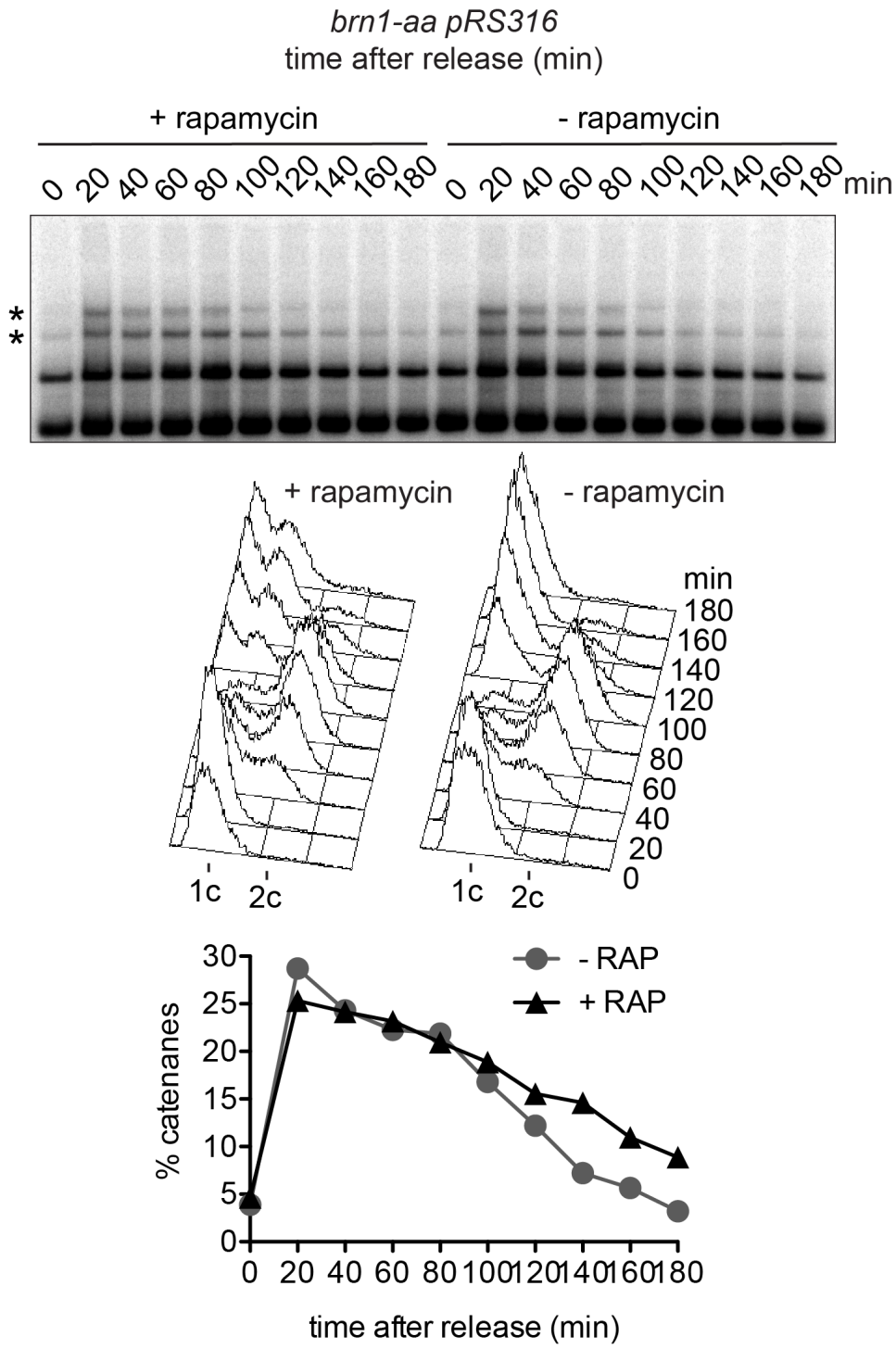

Figure 4.7 Condensin depletion has a smaller effect on pRS316 catenation

Cells of strain Y4333 were synchronised in G_1 and released into media containing rapamycin before re-arrest. Catenanes were quantified and plotted.

Compared to authentic chromosomes, pRS316 and pS14-8 are small in size and both contain only one replication origin. Given that catenation results from the meeting head-on of two replication forks during DNA replication, one can deduce that the number of topological links between catenated pRS316 and pS14-8 plasmids are few. In addition once a link is removed, if the plasmids are not held together by further links (or as we demonstrated, cohesin), then they will quickly physically separate and are unlikely to be recatenated by topoisomerase II. Given this, we hypothesised that condensin's contribution to decatenation would be greater in more complex topological landscapes than small minichromosomes. To address this prediction, we adapted our catenation assay to use a much larger substrate. In order to better represent the native chromosomes that this assay is modelling, we studied a 61 kb ring chromosome (RCIII, Figure 4.8), consisting of a 61kb section of *S. cerevisiae* chromosome 3, including the centromere, which has been excised then ligated to form a circular chromosome (Dershowitz and Newlon, 1993). In addition, this ring chromosome contained three origins of replication that would result in an increased number of topological linkages being formed between replicated DNAs during S-phase.

However, given the much larger size of this substrate, we found it much more difficult to separate its topological isoforms using the same purification and electrophoresis conditions as for pS14-8. Furthermore, owing to its much larger size, the ring chromosome was more susceptible to shearing during DNA purification. This resulted in a large fraction being linearised and creating greater background signal that made band quantification difficult.

4.2.1 Visualizing a ring chromosome's (RCIII) different isoforms

In order to address the difficulties in resolving this much larger ring chromosome, time was spent developing a new protocol to process the cell samples. In this new protocol, cells were suspended in agarose blocks in which they would then be lysed and processed to extract their DNA without any pipetting involved at all, following a specific protocol as described in (Jain et al., 2010). It was hoped that this gentler approach to DNA extraction would reduce or eliminate linearization of RCIII. In addition, the DNA from the agarose plugs would then be

resolved using Pulse Field Gel Electrophoresis (PFGE), a technique commonly used for DNAs of a large size. However, despite extensive troubleshooting the best results achieved (Figure 4.9) did not offer the resolution we hoped for. While it appeared that we had been successful in minimising the amount of linear forms generated (as none can be observed), the clarity of the different bands remained poor and offered little advantage over our previous DNA purification protocol. As such, we discontinued using PFGE and agarose gel plugs for DNA extraction and instead redesigned our previous DNA purification protocol to minimise the number of steps involving pipetting. In addition, we optimised the electrophoresis running conditions to give us the best resolution of isoforms possible, as described in chapter 2.4.

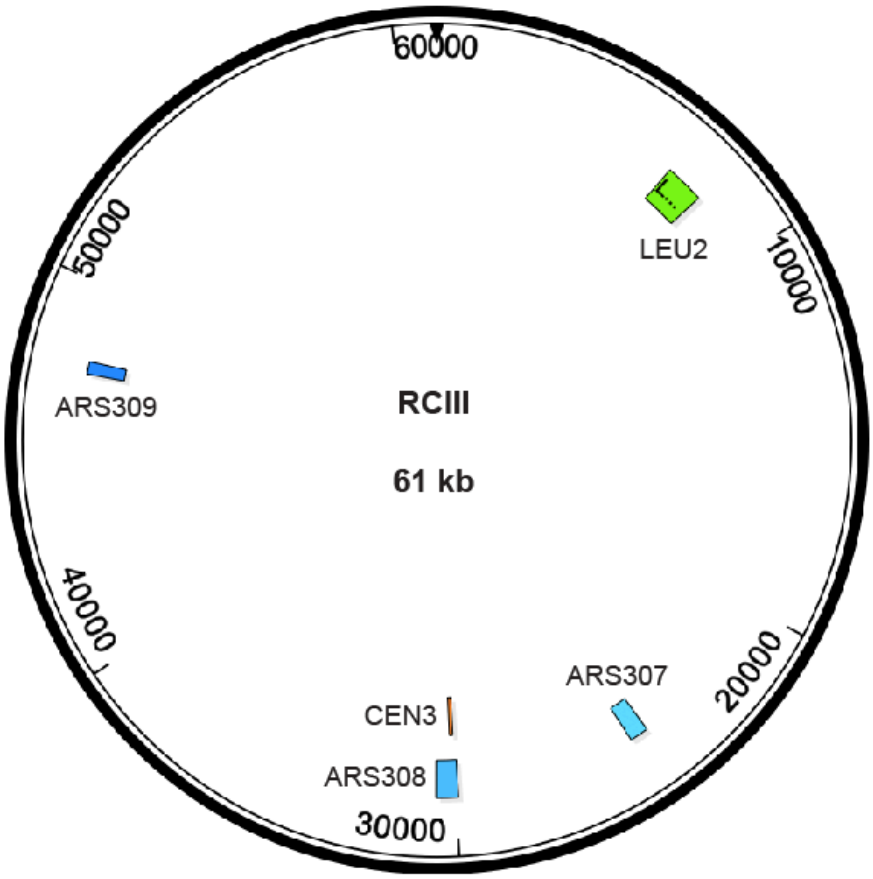

Figure 4.8 Map of ring chromosome RCIII

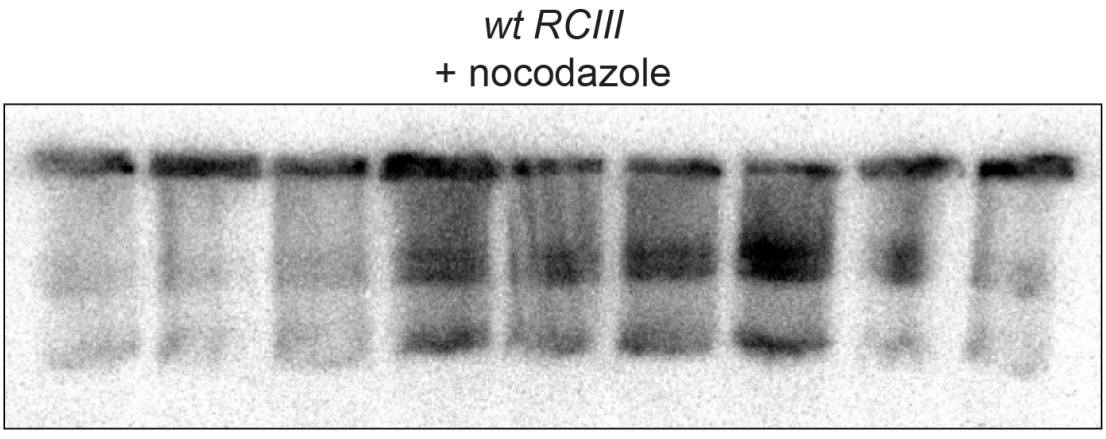

Figure 4.9 Resolution of RCIII by Pulse Field Gel Electrophoresis (PFGE)

Wild type cells (Y4259) were all metaphase arrested with nocodazole so that all isoforms would be present upon purification of DNA. Samples were digested in agarose plugs and DNA resolved by PFGE. However, despite eliminating shearing and linearization of RCIII, the resolution of individual isoforms is poor and are difficult to identify.

4.3 Condensin's decatenation role is more pronounced on RCIII

Having tried PFGE and returned to our original (albeit optimised) purification and electrophoresis protocol, we were able to find electrophoretic conditions that revealed wild type catenane formation during DNA replication (Y4259) (Figure 4.10). We were able to observe a discrete band appearing below the relaxed monomer (RM) band during DNA replication, that we identified as supercoiled catenanes (SC) via enzyme treatment. Incubation with topo I relaxed these species into a smear of relaxed and mixed catenanes (MC), probably of varied supercoiling status, migrating above relaxed monomers. Their catenated nature was confirmed by topo II treatment, which resolved these species into monomers. The general gel background in the region of mixed and relaxed catenanes, however, prevented their reliable analysis. In Figure 4.11 we see a quantification profile generated by the ImageQuant software. Given that many of the catenated forms run as a smear overlapping with bands representing monomers, the software is unable to correctly quantify each topological form. Despite this setback, we proceeded to perform several timecourse experiments using RCIII.

Focusing on the diagnostic supercoiled catenanes (SC), we observed their appearance in S-phase, followed by resolution at the time of mitosis. As with the smaller minichromosomes, only a fraction of the ring chromosome appeared catenated following S-phase. This suggests that a sizeable portion even of this large ring chromosome is decatenated rapidly following its replication. When we blocked mitotic progression using nocodazole, a similar level of catenanes was detectable during S-phase, but these were no longer resolved and persisted for an extended period in the arrest (Figure 4.12). The same was observed after condensin inactivation using the *brn1-9* mutation (Figure 4.13). In this case cells progressed through mitosis, yet catenanes following DNA replication persisted without sign of resolution for the remainder of the time course. A similar result was obtained following nuclear depletion of condensin (Figure 4.14). This suggests that two pools of catenanes are formed in S-phase. One pool is readily resolved following DNA synthesis, while a second pool of catenanes persists that is helped by condensin for their decatenation. The condensin requirement for resolution of this latter pool becomes more pronounced as chromosome size increases. In the

case of the 61 kb ring chromosome, resolution of persistent catenanes showed a strict condensin requirement.

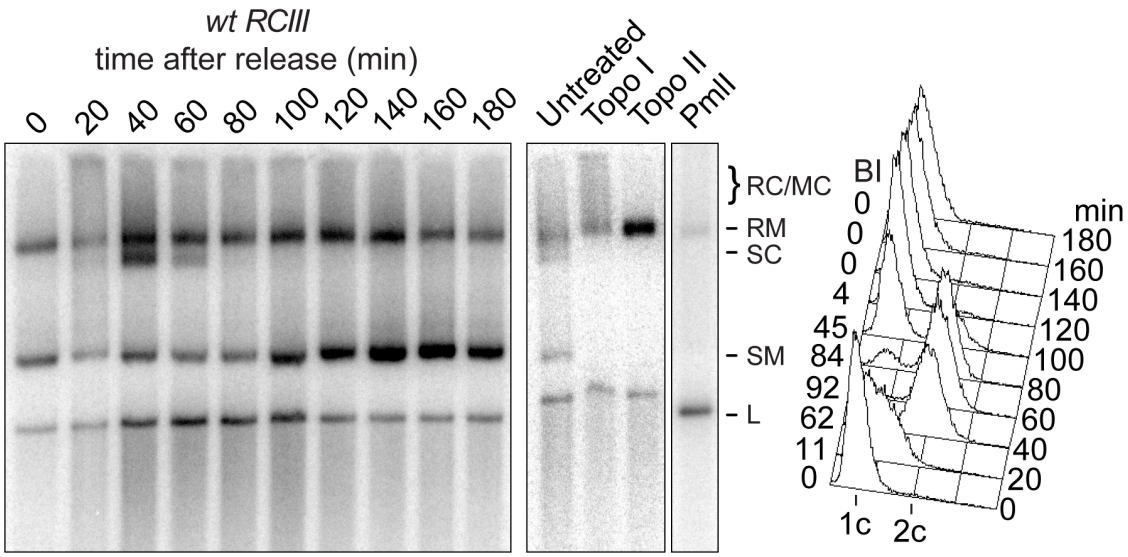

Figure 4.10 RCIII wild type timecourse and isoform identification

Wild type cells containing RCIII (Y4259) were synchronised in G_1 and then released to progress through one cell cycle before re-arrest in G_1. Enzyme treatments were used to determine which isoform each band represented; relaxed catenane (RC), supercoiled catenane (SC), mixed catenane (MC), relaxed monomer (RM), linear (L), and supercoiled monomer (SM).

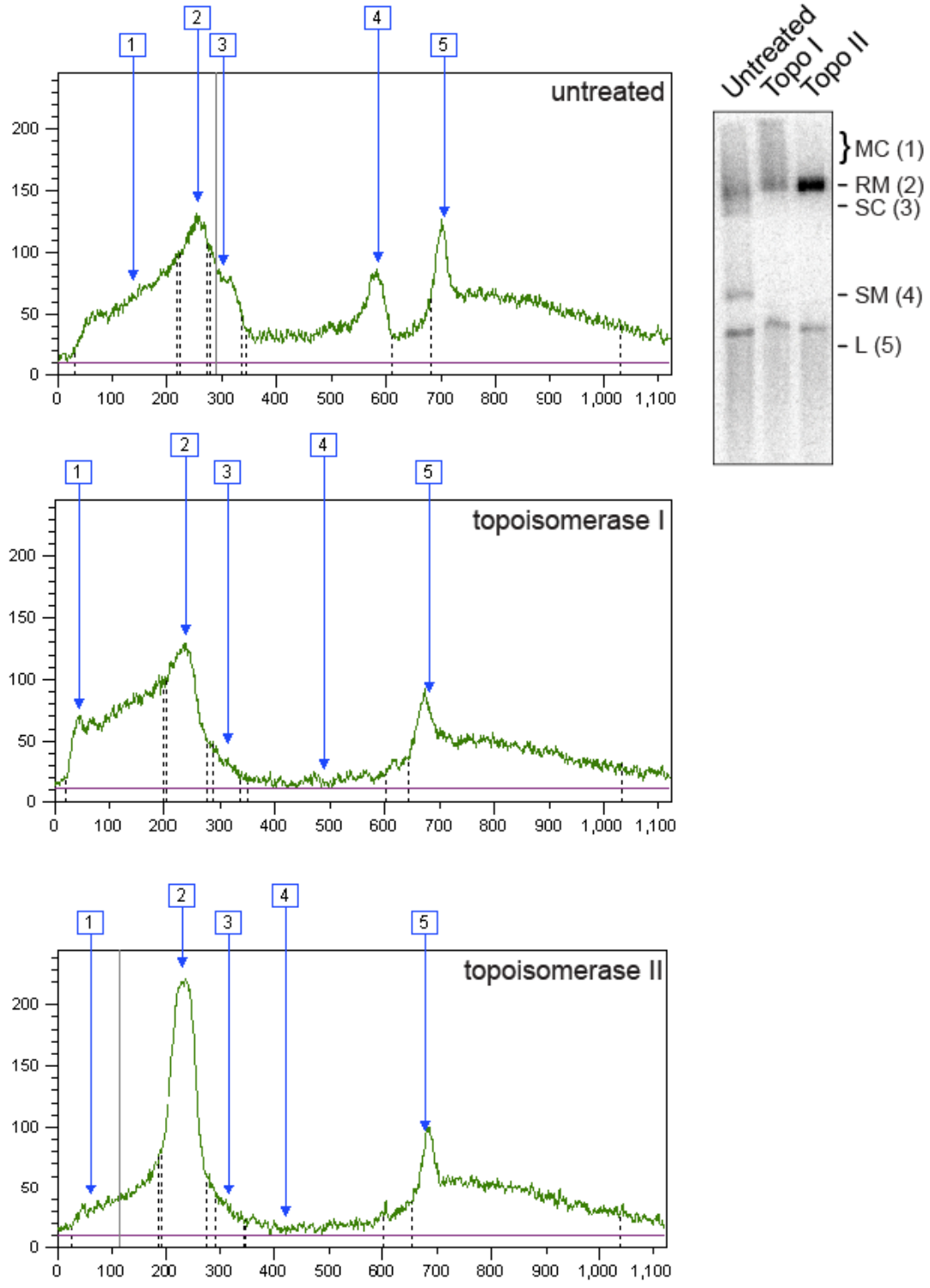

Figure 4.11 RCIII Quantification profiles

The three ImageQuant profiles above show the quantification profile for purified DNA samples that are untreated or treated with topoisomerase I or topoisomerase II. The profiles display the difficulty in quantifying catenanes as represented by (1) and (3) because of the large monomer peak (2) between them.

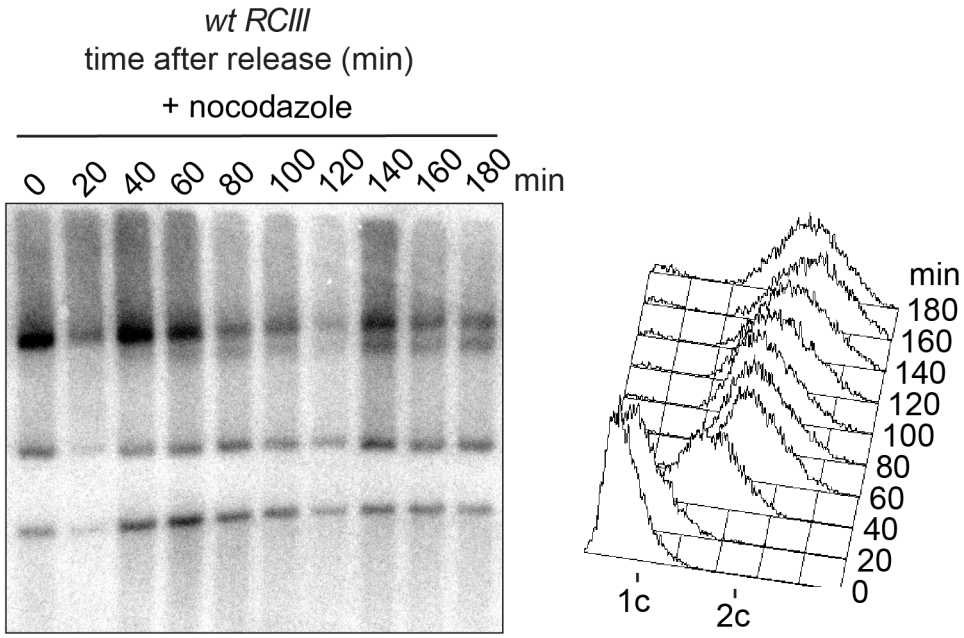

Figure 4.12 Nocodazole arrest shows persistent RCIII catenanes

Wild type cells containing RCIII (Y4259) were synchronised in G_1 and then released into media containing nocodazole causing metaphase arrest. DNA replication coincides with the appearance of a distinct catenated band.

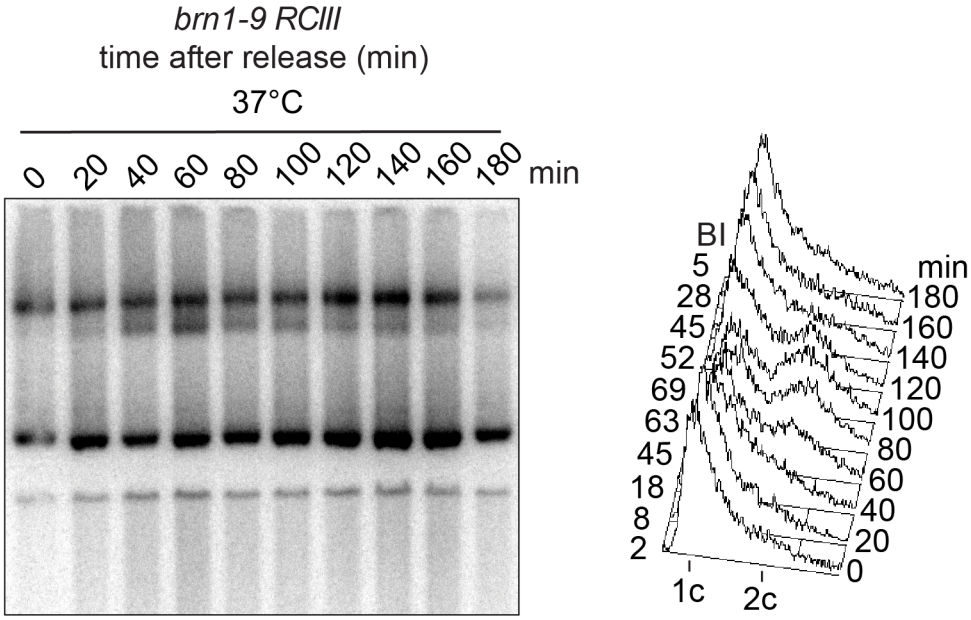

Figure 4.13 Condensin depletion phenotype closes matches nocodazole arrest

Brn1 was inactivated in RCIII containing cells (Y4264) by *ts* allele. Cells were synchronised in G_1 and then released into media at the non-permissive temperature and allowed to progress through one cell cycle before re-arrest.

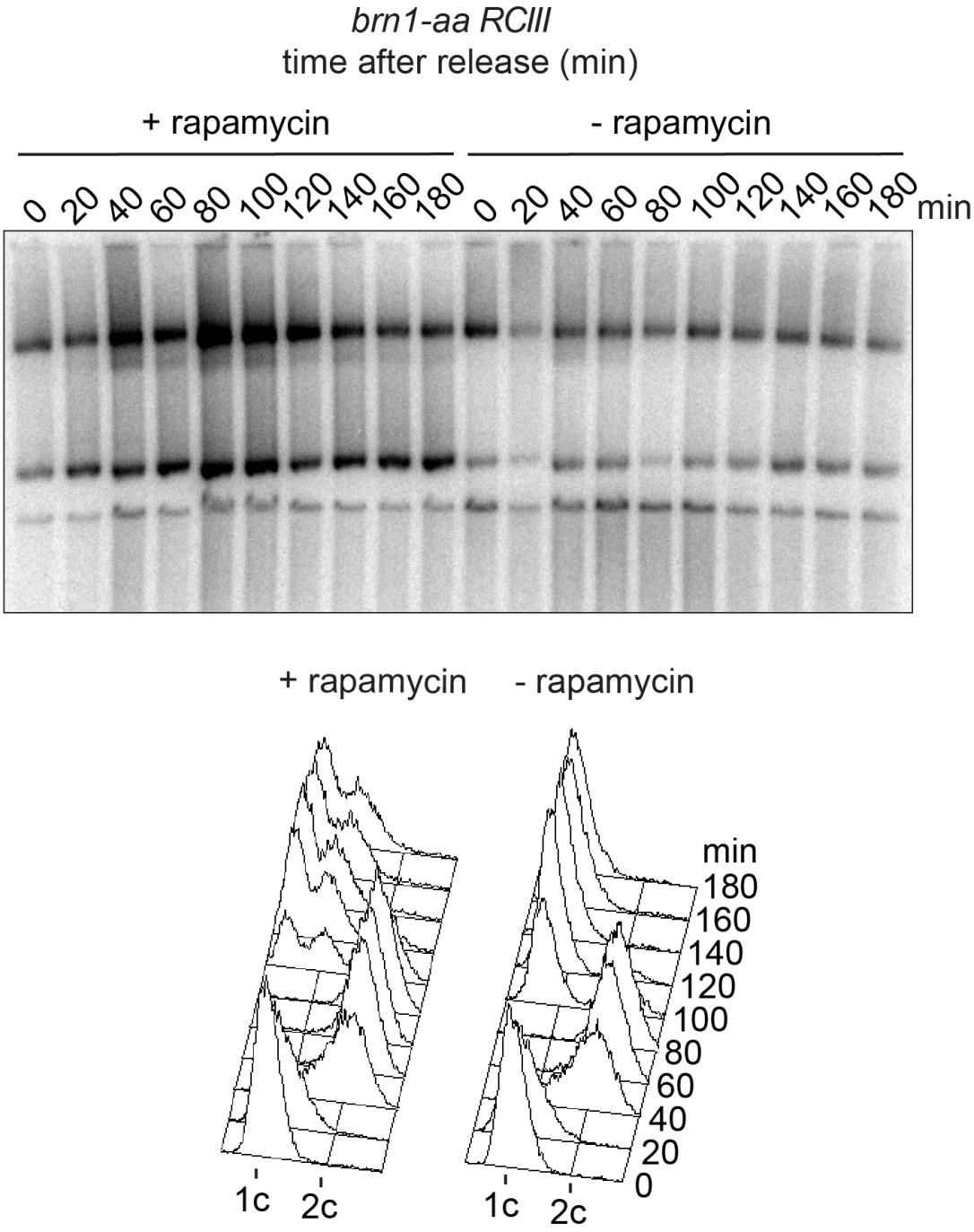

Figure 4.14 Nuclear depletion of Brn1

Brn1 was depleted from RCIII containing cells (Y4327) by anchor away. Cells were synchronised in G_1 and then released into media containing rapamycin and allowed to progress through one cell cycle before re-arrest.

At this point, we had shown that condensin is required for proper and complete removal of topological links between sister chromatids and consequently, depletion of condensin results in chromosome segregation defects. Therefore the next crucial issue to address was why this occurs. As discussed in the introduction, condensin itself is incapable of resolving topological linkages and this must be accomplished by the enzyme topoisomerase II. The question thus remains, how does the removal of condensin affect topoisomerase II, resulting in unresolved catenation? In the next results chapter, we will share the results of our investigations into the mechanics of the relationship between condensin and topoisomerase II.

Chapter 5. Results: Investigating Interactions

5.1 Do condensin and topoisomerase II directly interact?

In order to elucidate the mechanism by which condensin promotes sister chromatid decatenation, we asked whether we could detect a direct protein interaction between condensin and topo II. We fused the endogenous *TOP2* gene, encoding topo II, to a Pk epitope tag to facilitate immunoprecipitation and detection (Craven et al., 1998). The condensin subunit Brn1 was fused to an HA epitope. Brn1-HA was efficiently recovered in topo II-Pk immunoprecipitates, but not in a control immunoprecipitate from a strain lacking the topo II Pk tag (Figure 5.1A). This suggests that condensin interacts with topo II in yeast cell extracts.

However, doing the co-precipitation experiments by just using cell lysate could be generating false positives. As has been shown previously in the lab (D'Ambrosio et al., 2008b), the distribution of condensin and topoisomerase II is very similar along the DNA. Therefore, positive results for co-precipitation could be generated not by genuine interaction between the proteins, but instead by condensin and topoisomerase II both binding the same fragments of DNA in close proximity, effectively co-precipitating them via their association with the same stretch of DNA.

To overcome this potential false positive, we used a technique that more gently lysed the cells and allowed us to pellet the chromatin without fragmenting the DNA. In this way we collected the supernatant fraction, which would contain no DNA, and using this fraction only, tested for the interaction of condensin and topoisomerase II (Figure 5.1B). In this experiment, we now performed a Brn1-HA immunoprecipitation. However, in the HA pull-down, neither Brn1-HA nor Top2-Pk was recovered. It seems that protein stability was compromised in this type of extract.

To test this possibility, we returned to our original method of lysing the cells such that DNA was present in the whole cell extract and could mediate any possible interaction between the two proteins. However, we required an additional control to ensure that the original interaction observed was specific, and not just a consequence of the abundance of the two proteins. For this, we included the additional control of tagging cohesin (specifically Scc1) with the same HA epitope

tag that Brn1 was tagged with. By performing a topo II-Pk immunoprecipitation, we could see if the enzyme pulled down both proteins with it, or specifically just one (Figure 5.1C). Examining the results, we could again clearly detect condensin in the pull-down but we saw cohesin being pulled down as well. However, the single-tagged Scc1-HA6 control strain also exhibited a faint band in the pull down, indicating that there was some unspecific binding of cohesin by the beads. Despite further repeats, we were unable to get rid of the background cohesin binding by the beads, leaving us unable to make a clear decision on the nature of interaction between condensin and topoisomerase II. At this point, it seemed clear that we would be unable to obtain much more information from these sets of co-immunoprecipitation experiments, so we considered how else biochemically we could investigate their interaction.

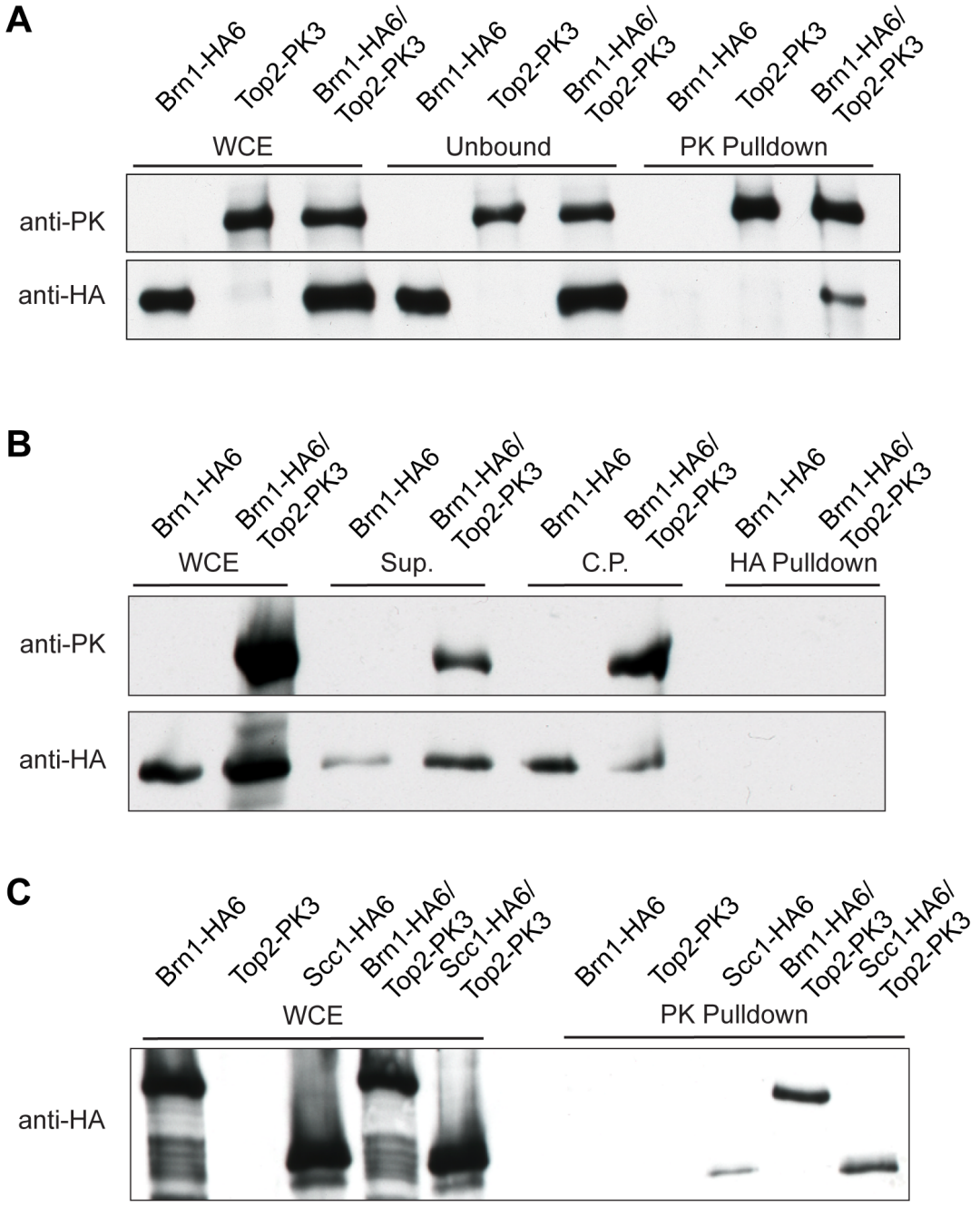

Figure 5.1 Co-immunoprecipitation of condensin and topoisomerase II

(**A**) Co-immunoprecipitation of condensin with topo II from yeast whole cell extracts. Topo II-Pk was immunoprecipitated using an antibody directed against its Pk epitope tag. Western blots of the input and unbound fractions, as well as the immunoprecipitates from strains containing the indicated epitope tag combinations are shown. (**B**) HA epitope pull-down was performed on cell lysate supernatant containing no DNA. (**C**) The same experiment as (A) but the extra control of Scc1-HA6 was included to verify if the interaction observed between condensin and topoisomerase II was specific.

5.2 Purification of topoisomerase II and condensin

To really be able to determine whether and how condensin and topo II interact, we decided to investigate whether biochemically purified condensin and topo II interact. To do this, we first purified topo II from yeast cells overexpressing the enzyme, following a published protocol (Worland and Wang, 1989). In Figure 5.2 we can see the steps of this effective purification technique, which yielded enzyme preparations in which topo II was the only protein detectable by Coomassie blue staining and was functionally active in trial decatenation and relaxation assays.

For the purification of condensin, the protein was affinity purified from yeast strain overexpressing the five condensin subunits (St-Pierre et al., 2009). Initially we attempted to purify condensin using the two-step purification protocol detailed in their paper, but had faced problems with low yields of condensin that consisted of subunits that were not stoichiometrically correct. In collaboration with Céline Bouchoux in the lab, we were instead able to purify condensin in a one step procedure using an anti-HA affinity matrix that specifically bound condensin.

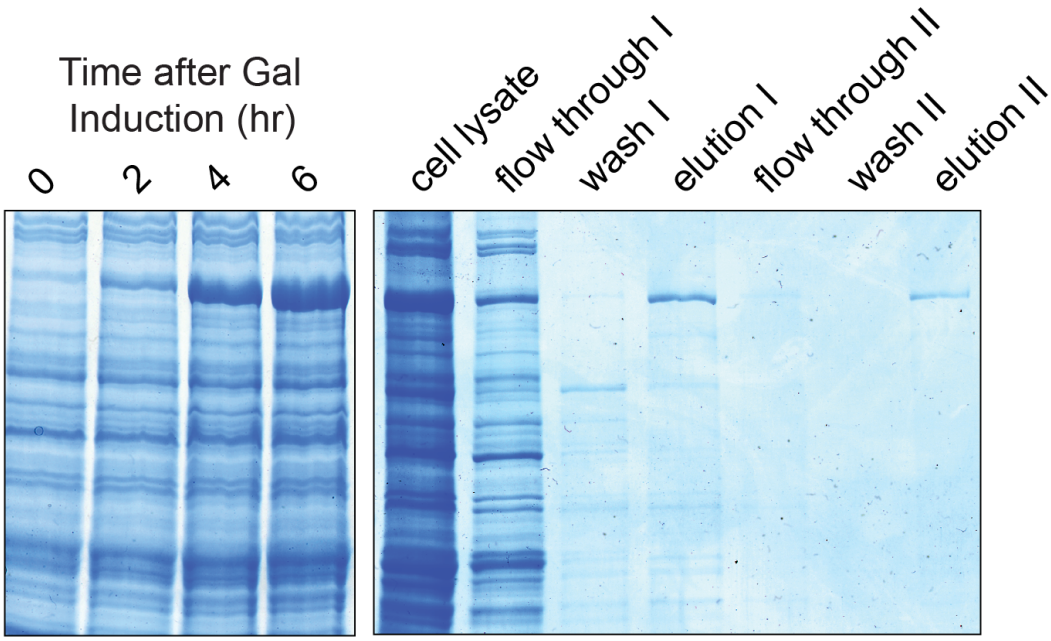

Figure 5.2 Topoisomerase II purification steps

Overexpression of topoisomerase II is induced through addition of galactose. After 6 hours, cells are collected, lysed and topoisomerase II is purified through a two-step process of binding and elution with cellulose phosphate and Q-sepharose.

5.3 Condensin and topoisomerase II directly interact

Having purified both protein complexes, we were now able to determine the nature of their interaction. To do this, condensin was bound via an HA epitope-tagged Brn1 subunit to α-HA antibody-coupled affinity beads, then purified topo II was added (Figure 5.3). Before performing the pull-down, both preparations underwent nuclease treatment. As a control, we added topo II to the α-HA affinity beads in the absence of condensin. After washes, bound proteins were eluted and analyzed. Topo II was efficiently recovered from condensin-bound, but not control, beads. This suggests that budding yeast topo II has a direct and physical interaction with condensin.

5.4 Condensin stimulates *in vitro* DNA decatenation by topo II

Given the physical interaction between condensin and topo II, we wondered whether condensin has a direct impact on the ability of topo II to resolve catenated DNA. As an assay to measure the decatenation activity of topo II, we used kinetoplast DNA (kDNA) as the substrate (Marini et al., 1980). kDNA is a mitochondria-derived network of intercatenated DNA minicircles of approximately 2.5 kb in size. Prior to decatenation, kDNA will remain in the wells during electrophoresis, owing to its high aggregate molecular weight. In the presence of topo II, decatenated minicircles are released that enter the gel. Incubation with our purified yeast topo II preparation led to efficient kDNA decatenation (Figure 5.4). DNA decatenation by topo II is an ATP-dependent reaction, and omission of ATP from the incubation prevented minicircle release. This serves as a control to ensure that we are observing topo II-associated decatenation activity and that gel entry is not due to a contaminating nuclease activity.

To study the effect of condensin on decatenation, we reduced the topo II concentration in the reaction such that, in the absence of condensin, approximately 10% of the kDNA substrate was decatenated during the incubation. Addition of increasing concentrations of condensin led to a marked, dose-dependent, stimulation of kDNA decatenation. In the presence of 20 nM condensin,

decatenation was stimulated by almost 3-fold compared to the reaction lacking condensin. Incubation of kDNA with condensin in the absence of topo II did not result in minicircle release. This suggests that condensin stimulates kDNA decatenation by topo II.

The reaction mix in which we observed the greatest stimulation of decatenation by condensin contained approximately equimolar amounts of kDNA, topo II and condensin. Considering that condensin and topo II interact, condensin might therefore directly stimulate the catalytic activity of topo II by binding to it. Alternatively, condensin might introduce a conformational change to the DNA substrate that facilitates its decatenation by topo II. To differentiate between these possibilities, we investigated whether the ability of budding yeast condensin to enhance kDNA decatenation is restricted to topo II from the same species, or whether it extends to type 2 topoisomerases from other organisms. We used commercial preparations of human topo IIα and *E. coli* topo IV in this comparison (Figure 5.5). Topoisomerase concentrations were again titrated such that in the absence of condensin approximately 10% of the kDNA substrate was resolved. Addition of condensin in each case led to a reproducible stimulation of the kDNA decatenation reaction. Human topo IIα shows 49% sequence identity and can functionally complement the essential *in vivo* function of yeast topo II (Jensen et al., 1996). A protein interaction between the two proteins could therefore be conserved between the species. This is less likely the case for *E. coli* topo IV, even though it's parE subunit shows 24% identity and 43% similarity to the N-terminal half of budding yeast topo II. Condensin might thus promote decatenation both by establishing a DNA substrate geometry that facilitates decatenation as well as recruit or activate topo II to assist the decatenation reaction.

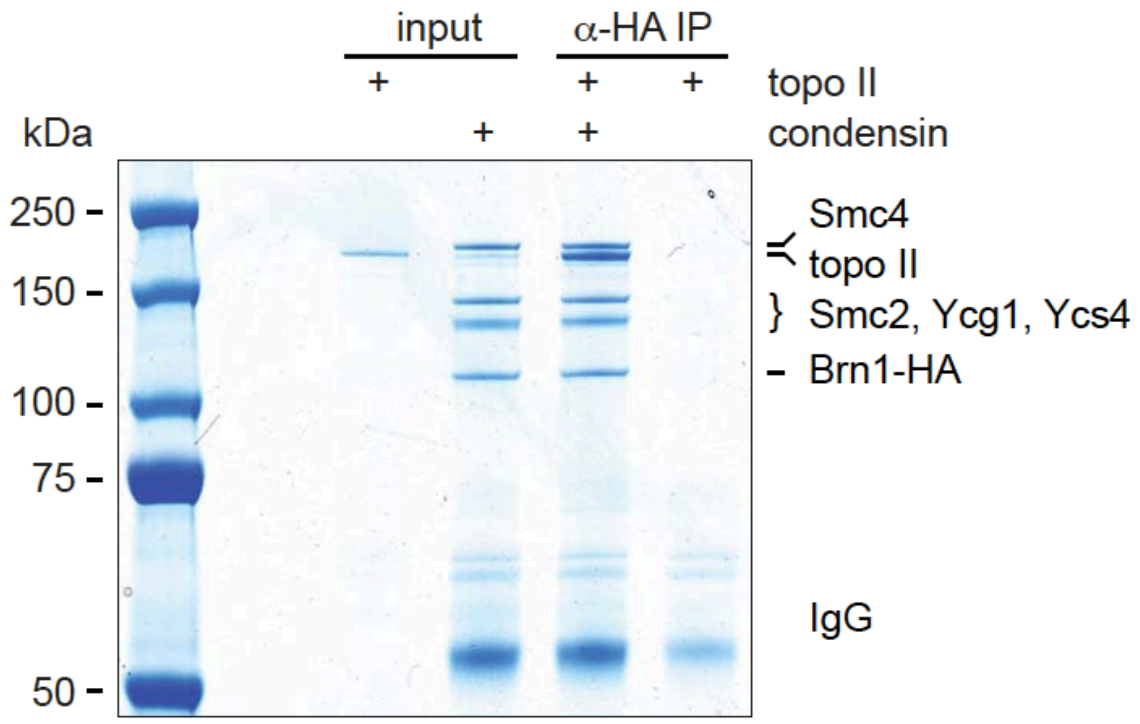

Figure 5.3 Condensin interacts directly with purified topoisomerase II

Purified topo II binds to condensin that is retained on an antibody affinity matrix. Purification and interaction analysis was performed as described in Materials and Methods, the input and eluates were analyzed by SDS-PAGE and proteins visualised by Coomassie blue staining.

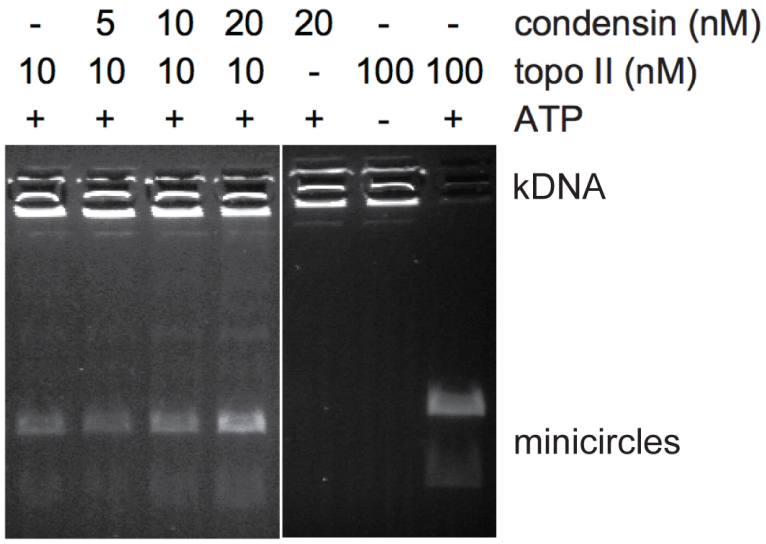

Figure 5.4 Condensin promotes decatenation of kinetoplasts

100 ng kDNA (corresponding to a concentration of 3 nM minicircles) were incubated with the indicated concentrations of purified yeast topo II and condensin. Reactions lacking topo II or lacking ATP were included as controls. Reaction products were resolved by agarose gel electrophoresis and visualised by ethidium bromide staining.

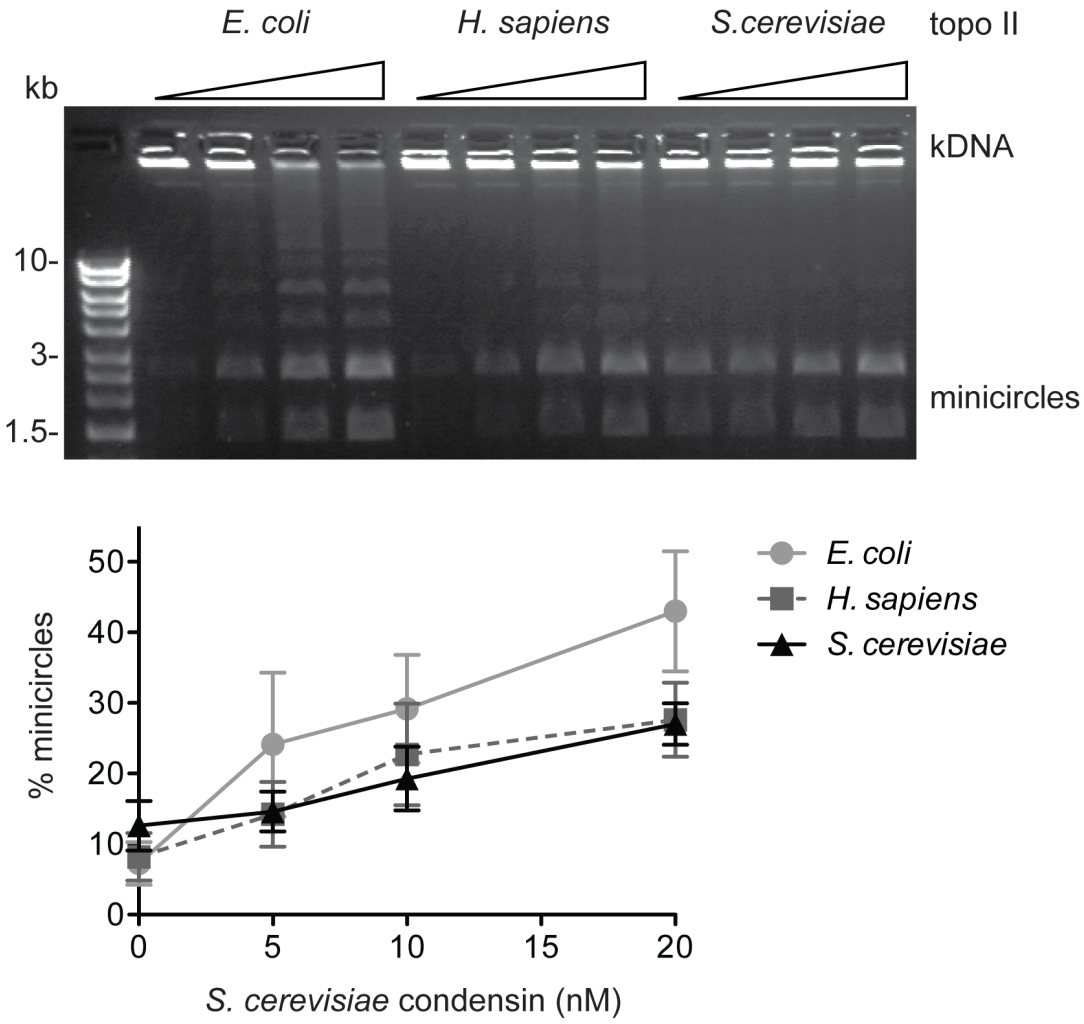

Figure 5.5 Condensin stimulates different topoisomerase II enzymes

Figure 5.5 as per Figure 5.4, but including reactions using *E. coli* topo IV and *H. sapiens* topo IIα. Minicircle release was quantified in at least 3 independent experiments with each topoisomerase. The mean and standard deviations are shown.

Chapter 6. Results: a-factor Synthesis and Characterization

6.1 Synthesis of a-factor

As was introduced in chapter 1.7, the anchor away strains we used were mostly mating type **α**, which meant that to synchronise the cells in G_1 the mating pheromone **a**-factor had to be used. However, while **α**-factor is readily produced by peptide synthesis laboratories in many research institutes as well as being commercially available, **a**-factor is not. This is because **α**-factor lacks any peptide modifications while **a**-factor has both farnesylation and carboxyl methylation, both of which are important for the biological potency of **a**-factor (Anderegg et al., 1988) (Marcus et al., 1991). This makes **α**-factor readily accessible by automated solid-phase peptide synthesis and much easier to manufacture. While over the years many **a**-factor synthesis strategies have been published, they typically involve liquid-phase reactions that are a hallmark of expert organic chemistry laboratories and thus out of reach for many users who do not have access to such expertise (Xue et al., 1989) (Sherrill et al., 1995) (Mullen et al., 2011).

In collaboration with the Peptide Synthesis laboratory at our institute, a novel strategy for the synthesis of **a**-factor was developed. The process is based on solid-phase peptide synthesis, followed by two simple steps in solution using widely available reagents and apparatus, thus making **a**-factor accessible to laboratories with standard peptide synthesis facilities (O'Reilly et al., 2012). Our contribution was to demonstrate the successful use of our synthetic **a**-factor to synchronise cell cycle progression of cells of the **α** mating type.

6.2 Use of a-factor to synchronise cells of α mating type

Initially we tried to synchronise haploid cells of the **α** mating type, using **a**-factor concentrations and conditions similar to what is used for **α**-factor synchronization of **a** mating type cells. Adding **a**-factor at a concentration of 0.5 µg/ml to asynchronously growing cells in early exponential phase led to efficient cell cycle arrest in G_1. However, upon filtration and washout of the **a**-factor, these

cells resumed cell cycle progression only later and very inefficiently. We therefore reduced the **a**-factor concentration through a series of optimisation experiments. Eventually we identified that addition of a total concentration of 0.04 µg/ml **a**-factor, in two halves at the beginning and after 1 h of arrest, efficiently arrested cells in G_1. Washout of the **a**-factor and resuspension in fresh pheromone-free medium now resulted in synchronous release from the arrest (Figure 6.1). We followed cell cycle progression by FACS analysis of DNA content, as well as by scoring the budding index and the fraction of binucleate cells. 60 minutes after release, when most cells showed a new bud, we added **a**-factor back to the culture to re-arrest cells after completion of mitosis in the following G_1. This analysis showed that arrest of α cells with **a**-factor and release by filtration gives rise to a cell population that transverses the cell cycle with very good synchrony.

The **a**-factor concentration we used in this protocol is 10-fold lower than the concentration of **2**-factor that we routinely use to synchronise **a** mating type cells. It is 100-fold lower than recommended **α**-factor concentrations in some synchronization protocols (Breeden, 1997). When we tried to use **α**-factor at a low concentration to arrest **a** mating type cells, we found that 0.04 µg/ml **α**-factor was not sufficient to impose a stable cell cycle arrest. We do not know the reason for the greater specific biological activity of **a**-factor as compared to **α**-factor. Its hydrophobic nature and farnesylation might enhance its affinity for lipid membranes and thereby increase its local concentration close to the target cell **a**-factor receptor. In addition, the absence of a known **a**-factor protease, equivalent to the Bar1 **α**-factor protease, may mean that **a**-factor is more stable in the culture medium or in the vicinity of the receptor.

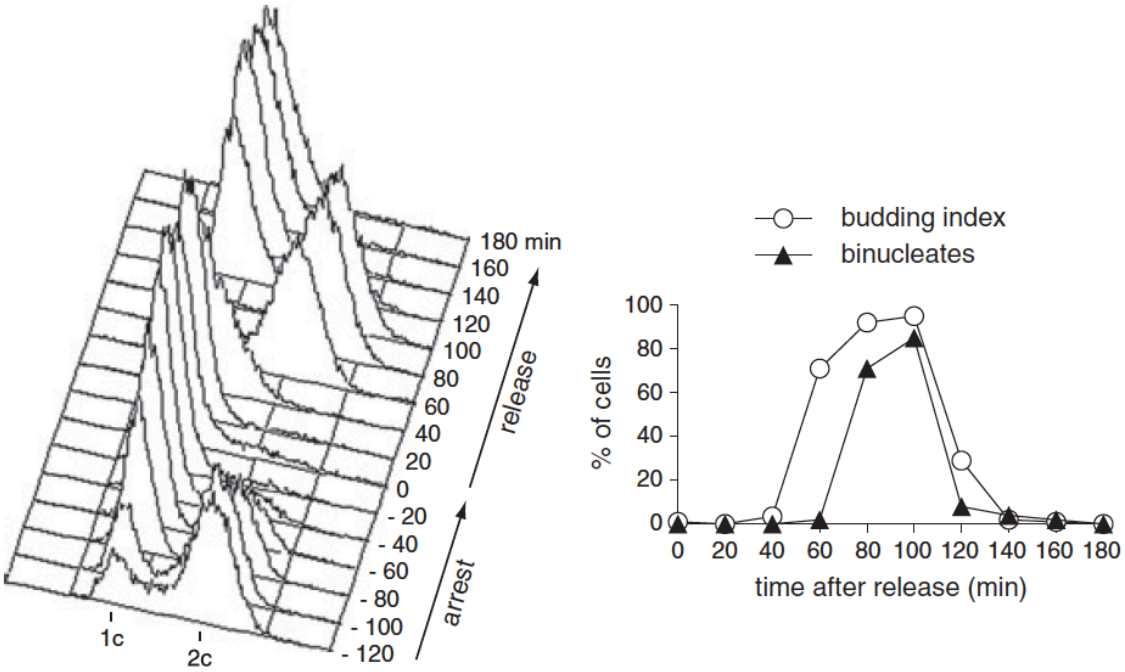

Figure 6.1 Use of a-factor to synchronise cell cycle progression of α cells

0.02 µg/ml **a**-factor was added to a culture of exponentially growing budding yeast cells of **α** mating type. After 60 minutes, a second 0.02 µg/ml dose of the pheromone was added. After 120 minutes, all cells were arrested in G_1 (time point 0). The culture was then filtered and the cells were washed and resuspended in fresh medium lacking **a**-factor. The cells now passed through a synchronous cell cycle and **a**-factor was added to the culture again after 60 minutes to impose re-arrest in the following G_1. Cell cycle progression was monitored by FACS analysis of DNA content and microscopic analysis of the budding index and binucleate cells.

6.3 Little shmoo formation during *a*-factor-induced G_1 arrest

When we microscopically followed the **a**-factor response during cell synchronization, we noticed that even though cells arrested unbudded, they showed hardly any signs of shmoo formation. This was unlike **α**-factor-treated **a** mating type cells, where G_1 arrest is typically accompanied by noticeable shmoo formation (Figure 6.2). In our cultures, **α** cells treated with synthetic **a**-factor lost their oval shape and took on a more rounded, sometimes lemon-shaped, appearance. The cell volume increased over time in the arrest, but directed shmoo formation was barely observed. This was even the case at higher pheromone concentrations, similar to those that elicit efficient shmoo formation of **α**-factor-treated **a** mating type cells (Figure 6.2). While it has been previously noticed that **α** cell shmoos differ from the typical **a** cell projections (Wilkinson and Pringle, 1974) (Betz et al., 1977), our observations suggest that as yet poorly understood differences exist in the way the opposite mating pheromones act on yeast cells, or in the way that cells of the opposite mating types react to pheromone exposure.

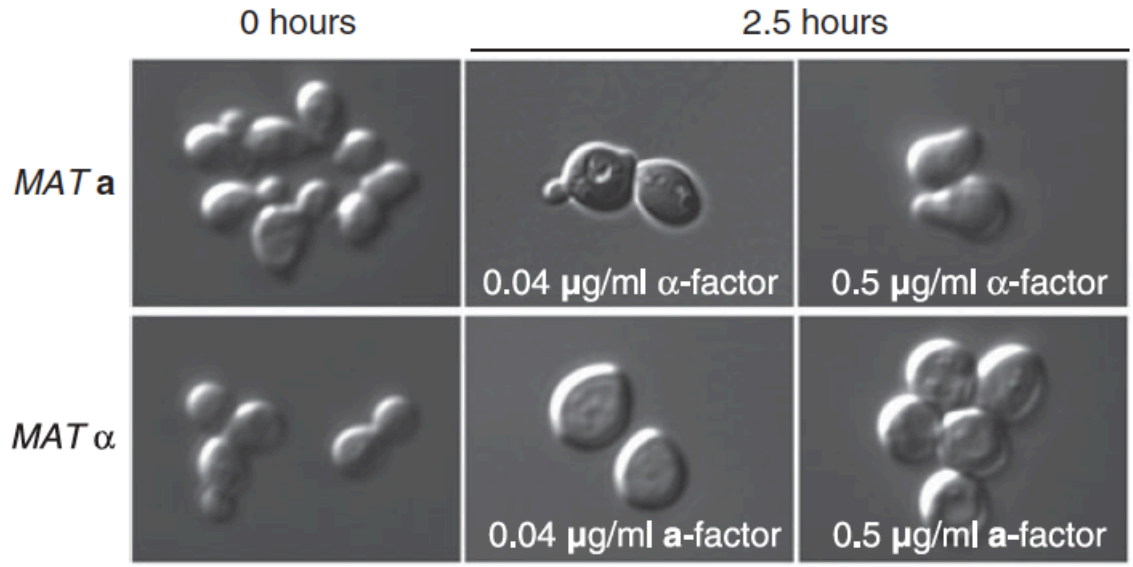

Figure 6.2 Comparison of pheromone-induced shmoo formation of a and α cells

Haploid cells of either **a** or **α** mating type were grown in early exponential phase. Mating pheromones **α**- and **a**-factor, respectively, were added at the indicated concentrations. Cells were photographed before and 2.5 hours after pheromone addition, using a Zeiss Axioplan 2 microscope equipped with differential interference contrast optics.

Chapter 7. Discussion

7.1 Catenation can be observed in minichromosomes

In our experiments, we have shown the utility of our catenation assay in observing and tracking catenanes for a range of minichromosome sizes. Despite previous reports that replication-dependent catenation of smaller minichromosomes was too transient to be detectable in wild type cells (Baxter et al., 2011), we could observe catenation with all our minichromosome substrates. By careful optimisation of our DNA purification process and electrophoresis conditions, we established parameters that enabled us to clearly track catenane behaviour through the cell cycle and our purified minichromosomes show distinct patterns of replication-dependent catenation that resolve with a specific timing.

The significance of our assay results was further supported by the dual approaches we took towards protein inactivation. By being able to inactivate proteins by both *ts* allele disruption and anchor away-mediated nuclear depletion, we were able to verify that the resultant phenotypes were indeed a specific result of the target protein's inactivation.

7.2 Cohesin protects catenation

When cohesin was inactivated through either use of a *ts* Scc1 allele or nuclear depletion via anchor away, we saw a significant reduction in the abundance of catenanes. During S-phase, when we normally see the greatest proportion of catenanes, less than 20% of minichromosomes were detected as catenated. Thus it appears that cohesin plays a substantial role in the protection of catenation. There are differing mechanisms by which cohesin could exert this influence; an 'indirect' mechanism whereby cohesin might retard topo II-driven decatenation hence maintaining topological linkages, or 'directly' through the entrapment of sister DNAs within the cohesin tripartite ring. In the former mechanism, cohesin could retard decatenation by topo II through some sort of interaction. In our co-immunoprecipitation studies in chapter 5.1, we used cohesin as a control and saw an unexpected interaction between Scc1 and Top2, which suggests that some

direct interaction between the two proteins is at least feasible. Another possibility is that the cohesin complex blocks topo II's access to sites of intertwining, inhibiting decatenation. As the cohesin tripartite ring physically entraps the sister DNAs, it is possible that the sites of entrapment coincide with where the sister DNAs are physically closest, or catenated. The presence of cohesin could therefore block access until the time it is cleaved by separase.

However, if we take into account the effect of other mitotic components on catenation, it seems that cohesin may play a more indirect role. If we compare catenane levels between cells arrested in metaphase either by nocodazole or by inactivation of Cdc20, we can see that there is a marked decrease in catenanes when the spindle is present. In both situations cohesin is still present and uncleaved, yet the presence of the mitotic spindle seems to promote decatenation, implying that the cohesin complex isn't blocking topo II. Thus, cohesin could be playing a more direct role in maintaining catenation. We hypothesize that while topo II is able to access and act upon the sister DNAs, prior to cohesin cleavage, those sister DNAs will remain physically intimate owing to their entrapment by the cohesin ring. Therefore, the reintroduction of topological linkages owing to the bi-directionality of topo II is possible. As long as cohesin remains uncleaved, an equilibrium of decatenation and recatenation will be established between sister DNAs. The influence of other factors, such as the tension exerted by the mitotic spindle or the cleavage of cohesin allowing sister DNAs to move away from each other, will alter the balance of that equilibrium. A recent study has come to a similar conclusion on cohesin's catenane-protection properties (Farcas et al., 2011).

7.3 Anaphase bridges result from persistent sister chromatid catenanes

Chromosome segregation failure and anaphase bridges are a hallmark phenotype of cells with compromised condensin function, yet prior to our study, the reason underlying this failure has remained undetermined. We now provide evidence that, at least in the case of circular minichromosomes in budding yeast, condensin is required to promote complete resolution of catenanes that are retained between sister chromatids following their replication. Persistent sister

chromatid catenanes have previously been proposed as a source for anaphase bridges in a condensin mutant (Bhat et al., 1996), though it is to our knowledge the first time that these catenanes have been directly observed. An important prediction from a model where sister chromatid catenation prevents chromosome segregation is that ectopic decatenation should resolve the anaphase bridges in the absence of condensin. This has been achieved in case of the budding yeast rDNA locus where anaphase bridges in a condensin mutant were resolved by ectopic expression of a viral topo II enzyme. Though again the catenations status of the locus could not be observed at the time (D'Ambrosio et al., 2008a). Taken together with the direct visualization of persistent catenanes between circular chromosomes that we now report, we can suggest the chromosome bridges that arise when condensin function is compromised are indeed due to persistent DNA catenation.

7.4 Condensin promotes decatenation by topoisomerase II

An important area for future research is to investigate whether catenation between linear chromosomes is regulated in the same way as we have observed here in the case of circular substrates. Catenation, in theory, should not exist between linear pieces of DNA, as any intertwining could easily slide off the DNA ends. This is a reason why catenation of linear chromosomes cannot be studied after DNA isolation by gel electrophoresis. *In vivo*, however, chromosome movement is constrained such that catenation between linear sister chromatids is maintained and has the ability to restrain chromosome segregation during anaphase (Holm et al., 1985, Uemura et al., 1987b). Whether, and how far, topological links between sister chromatids might be able to translocate along DNA strands is not known. Helical tension along individual sister strands is constrained to regions of approximately 100 kb in budding yeast (Joshi et al., 2010). It seems likely that sister chromatid catenation is similarly constrained. Topologically isolated domains along chromosomes are probably the consequence of protein-mediated DNA interactions, as has been demonstrated at least in the case of mitotic chromosomes (Kawamura et al., 2010). How topological interlinks between linear

chromosomes arise, where and for how long they are maintained, and how they are eventually resolved are important question. To address these will likely require the topological isolation of sections of linear chromosomes by excision in a circular form to facilitate their analysis.

7.5 Decatenation occurs in steps

While studying the sister chromatid catenation status of circular chromosomes during the cell cycle, we found that most catenanes are rapidly resolved by topo II soon after DNA replication. When topo II was active, at most half of the chromosomes were seen catenated in our synchronised cell population at any one time. Only 10-20% catenanes persisted until mitosis, or in the absence of cohesin when proteinaceous sister links were removed. This fraction is similar to the fraction of catenanes that persisted throughout mitosis and into G_1 of the next cell cycle in cells depleted of condensin. This raises the question whether there is a distinction between catenanes that are readily resolved and those that are decatenated later in the cell cycle and require condensin for their resolution? We can see two possible scenarios. In the first, all replication products are left catenated in the same way, possibly by a small number of interlinks per replication termination site. Topo II will act on these based on chance encounters sister DNA strands. Based on its mode of DNA binding, topo II favors decatenation over catenation at such crossing points (Vologodskii et al., 2001, Dong and Berger, 2007). Resolution is limited in this scenario by Brownian movement of sister chromatids, constrained by cohesin-dependent cohesion. Condensin's role would be to accelerate resolution by stimulating topo II activity and promoting a substrate geometry conducive to decatenation. In an alternative scenario, topological interlinks between sister chromatids might exist in different forms. Depending on the reactions involved in replication termination and constraints imposed by the chromosome environment the catenanes produced might differ in the number of interlinks or additional topological features, e.g. knots. Simple catenanes could be good substrates for rapid decatenation, while condensin would aid the resolution of more complex topologies. In *S. cerevisiae*, the rDNA shows the greatest resolution problems in condensin mutants (D'Ambrosio et al., 2008a). This area of DNA is

highly transcribed, even during mitosis, possibly generating topologically complex catenanes that benefit more from condensin activity. Condensin binding to DNA introduces positive-handed writhe, which in the presence of topo II allows knot formation (Kimura et al., 1999). Equally, by stabilizing transient DNA conformations that rarely form spontaneously, condensin might facilitate resolution of complex structures. In support of this possibility, the fraction of catenanes that require condensin for resolution appear to be refractory to decatenation in its absence and persist for extended time periods. Such structures might also pose the greatest danger to chromosome segregation.

7.6 Condensin directly interacts with topoisomerase II

How does condensin promote sister chromatid decatenation? As discussed above condensin might act at the level of the DNA substrate to facilitate catenane resolution, maybe in particular those of a complex topological nature. Our observation that budding yeast condensin facilitates the *in vitro* decatenation of kDNA circles not only by budding yeast topo II but also by topoisomerases of human and bacterial origin is consistent with such an activity. In addition to affecting DNA conformation, we also observed a direct protein interaction between budding yeast condensin and topo II. Such an interaction could directly stimulate the catalytic activity of topo II as has been recently observed in the bacterial system (Li et al., 2010, Hayama and Marians, 2010). Yet another way how condensin's interaction with topo II could facilitate sister chromatid decatenation is by recruitment of topo II to its sites of action. Condensin and topo II show significant overlap in their chromosomal association pattern (D'Ambrosio et al., 2008b), consistent with a role in topo II recruitment.

7.7 Future perspectives

Our catenation assay has proved to be a useful and flexible tool in our investigation, having produced an array of interesting results. Coupled with our *in vitro* biochemical experiments using purified proteins, we have been able to make some novel insights into condensin's and topoisomerase II's role in chromosome

resolution. However, looking to the future we believe that the next logical step would be to move on from using minichromosomes as models and to develop techniques to study catenation on the native chromosomes themselves. We believe that we have reached the limit of what can be accurately observed using minichromosomes. In our experiments, as minichromosome size increased so did linearization of the substrate, most likely owing to an increased vulnerability to pipette-induced shearing. We found that by minimizing the steps in our purification protocol that involved pipette use, as well as implementing other purification optimizations, we could minimise the amount linearised. However, it is likely that our assay has pushed the limits of what is observable with large sized minichromosomes. RCIII, being over 60 kb in size, already presented many difficulties in resolution and was very susceptible to damage. While we found that pulse field gel electrophoresis did not yield satisfactory resolution for our assay, it is likely that any larger substrates would have to be resolved via this technique. However, we feel that the next logical step for future investigations would be the formation of a technique that could manipulate the native chromosomes themselves, a direction that some labs have already tentatively begun moving in (Farcas et al., 2011). It is feasible that native chromosomes could be engineered such that sections could be cut out and ligated *in vivo* to create circular DNAs that are easily assayable by adapting our current protocols, perhaps through adaption of the Cre/lox recombinase system commonly used in mammalian and plant cells (Figure 7.1). With the availability of condensin binding maps, replication origins and knowledge of areas of potential catenation, interesting sites could be marked for excision and study finally allowing for the direct observation of endogenous chromosomes.

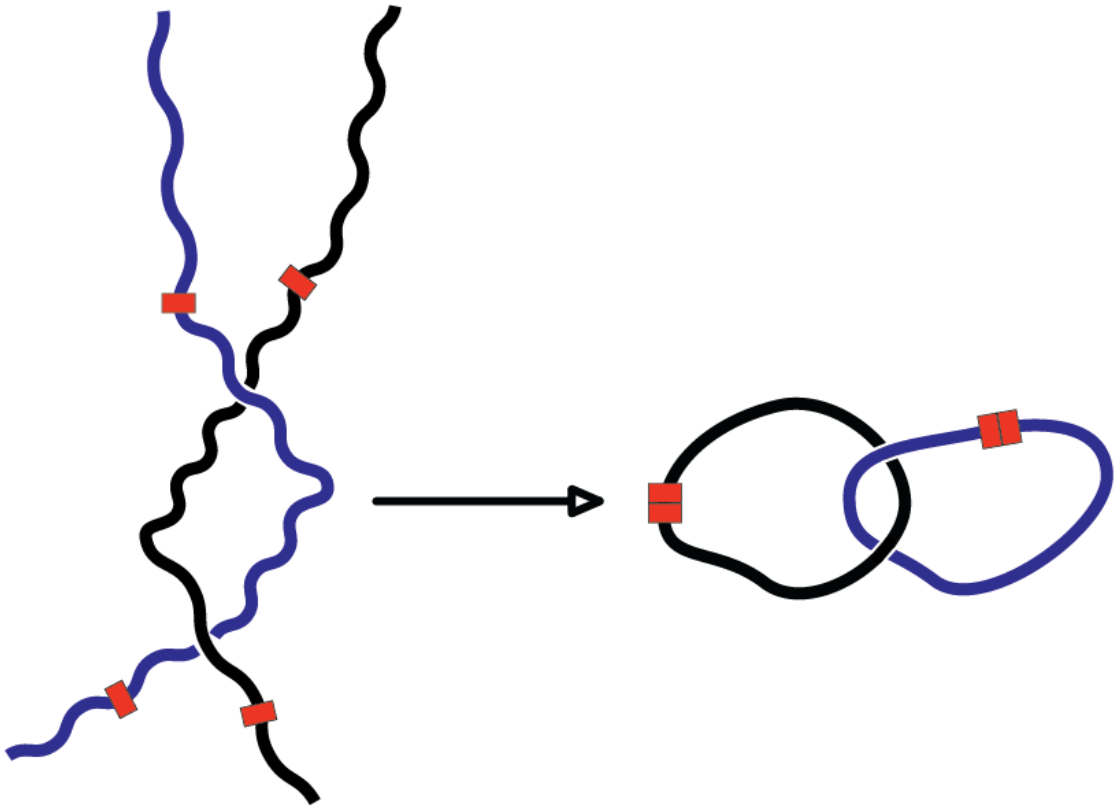

Figure 7.1 Schematic displaying possible excision and re-ligation of endogenous chromosome DNA to form *de novo* ring chromosomes *in vivo*

In the figure two sister DNAs are topologically linked and cut sites (red boxes) flank the site of catenation. Upon excision at these cut sites and consequent ligation, topologically linked circular DNAs are created.

We are still very naïve about how cells deal with the immense topological challenge of replicating millimeter-long chromosomes in micrometer-sized nuclei and ensuring the smooth resolution of DNA interlinks during the segregation of sister chromatids during mitosis. Cell division underpins the propagation of all life on earth and unraveling this fundamental process is both fascinating and essential. Chromosome resolution and segregation lies at the heart of human growth, reproduction and regeneration and the danger of persisting anaphase bridges to genome stability makes this an important area of further study.

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